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Teoria do Mosaicismo Cólico

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«With ≈ 10^{13} to 10^{14} cells in a body, and probably >10^{16} cells produced over a lifetime, the lineage history within a single individual is much greater than for all hominids that have ever lived, perhaps as great as for all the primates that have ever lived.»

Steven A. Frank

Resumo

O cancro colo-rectal (CCR) continua a ser uma das principais causas de morte por cancro nos países desenvolvidos. No entanto, o CCR pode ser prevenido através da remoção das suas lesões precursoras - os adenomas. Diversos estudos demonstraram já que esta atitude reduz a mortalidade por CCR e que as características dos adenomas na primeira colonoscopia permitem definir estratégias de vigilância.

Sabe-se atualmente que diferentes vias de carcinogénese podem conduzir ao CCR esporádico, evoluindo ao longo de anos numa mucosa macroscopicamente inalterada. Alguns dados apontam para que os primeiros eventos nestas sequências de carcinogénese possam remontar à embriogénese e afetar apenas determinadas áreas do cólon, derivadas de células precursoras mutadas. A acumulação posterior de outras mutações vai culminar na primeira manifestação clínica – o adenoma, numa janela temporal limitada.

Numa análise de doentes com adenomas esporádicos, demonstrámos que sob ação da polipectomia, a incidência de adenomas decai ao longo do tempo, sugerindo um efeito de eliminação de todos os clones derivados de células precocemente mutadas.

Em doentes com CCR e adenomas síncronos, demonstrámos uma co localização das lesões que favorece a noção de alguma forma de mosaicismo cólico.

Finalmente, numa amostra de doentes com CCR avaliada prospectivamente, confirmámos a diversidade de vias de carcinogénese que culminam no mesmo evento final e a importância da avaliação molecular e genética para a sua distinção, com implicações clínicas.

Apesar de não ter sido possível demonstrar a base genética do mosaicismo cólico, defendemos que os dados acumulados até ao presente continuam a apontar nesse sentido e que o conhecimento aprofundado da origem celular e sub-celular do CCR será o caminho para a sua erradicação.

Palavras-chave:

Cancro Colo-Rectal.
Adenoma.
Mosaicismo.
Carcinogénese.

Abstract

Colorectal cancer (CRC) remains one of the main causes of death by cancer in developed countries. Yet, it is potentially preventable, by removing the precursor lesions - adenomas. Several studies proved that this intervention reduces CRC mortality and that the adenomas' characteristics at the first colonoscopy can guide surveillance strategies.

It is now known that several carcinogenesis pathways may lead to sporadic CRC, evolving for years in macroscopically unchanged mucosa. Some data points to an embryonic origin for the first events in these pathways, affecting only some colonic areas, derived from mutated precursor cells. The latter accumulation of other mutations then leads to the fist clinical manifestation – the adenoma, in a limited time frame.

In a sporadic adenomas patient population, we showed that under polypectomy the adenoma incidence decays along time, suggesting an effect of the elimination of all clones derived from early mutated cells.

In CRC patients with synchronous adenomas, a co-localization of lesions was found, favoring the notion of some form of colonic mosaicism.

Finally, in a prospectively evaluated CRC patient sample, we confirmed the diverse carcinogenesis pathways leading to the same final event and the importance of molecular and genetic studies to distinguish them, with clinical implications.

Although we could not prove the genetic basis of the colonic mosaicism, we state that all accumulated evidence still points to it and that the deeper knowledge of the cellular and sub-cellular origin of CRC will be the way to its eradication.

Key-words:

Colorectal Cancer.
Adenoma.
Mosaicism.
Carcinogenesis.

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Nota: No texto desta tese foi utilizado o novo acordo ortográfico, exceto na palavra «recto», que no campo da Medicina continua a ser utilizada com a grafia anterior.

1. Introdução

1.1. A importância do cancro colo-rectal

Segundo os dados da Organização Mundial de Saúde (OMS), em 2012 ocorreram mais de 8 milhões de mortes por cancro, em todo o mundo.[1] O cancro colo-rectal (CCR) foi o terceiro cancro mais comum no sexo masculino e o segundo mais comum no sexo feminino (10% e 9% do total, respetivamente). [1,2]

Em 2012, Portugal ocupava o décimo quarto lugar na Europa em termos de incidência de CCR.[1] Os dados portugueses do Registo Oncológico Regional do Sul, que inclui as regiões de Lisboa e Vale do Tejo, Alentejo, Algarve e Região Autónoma da Madeira mostravam, em 2009, uma taxa de incidência bruta de 48,5 para o cólon e de 24,3 para o recto.[3]

Nos Estados Unidos da América (EUA), a probabilidade cumulativa de um diagnóstico de CCR ao longo da vida é de 4,7% no sexo feminino e 5,0% no sexo masculino. [4]

A incidência de CCR exibe grande variação regional em termos mundiais, com cerca de 55% dos casos a ocorrerem nas regiões mais desenvolvidas.[1] No entanto, nestas regiões a mortalidade por CCR é inferior à incidência, verificando-se uma sobrevivência claramente inferior nas regiões menos desenvolvidas do globo, onde ocorrem 52% do total mundial das mortes por CCR, apesar da menor incidência.[1]

Esta clara diferença em termos de mortalidade deve-se, não só a uma maior disponibilidade de terapêuticas eficazes nos países mais desenvolvidos, mas também à implementação de programas de rastreio.[5]

Os programas de rastreio permitem, não só identificar CCRs em estádios mais precoces, mas também identificar e remover (através da polipectomia) as suas lesões precursoras, os adenomas. No *National Polyp Study*[6,7] ficou demonstrado que a colonoscopia com polipectomia reduz claramente a incidência de CCR. Aliás, só este efeito dos programas de rastreio na eliminação das lesões precursoras permite explicar a redução sustentada da mortalidade por CCR, até 10, 15 ou mesmo 30 anos de seguimento, consoante os estudos.[5,8,9]

O facto de as colonoscopias de rastreio se associarem a uma maior redução do risco subsequente de CCR quando comparadas com as colonoscopias realizadas por outras indicações[9] também aponta para a eliminação de lesões precursoras como a maior vantagem dos programas de rastreio.

A eficácia dos programas de rastreio espelha-se na redução da incidência de CCR de 3,4% por ano ao longo da década 2001-2010 nos EUA, em paralelo com um aumento do rastreio por colonoscopia de 19% para 55%.[4]

Em suma, o CCR é um dos maiores problemas de saúde a nível mundial, mas pode ser combatido através da prevenção, nomeadamente com a deteção e remoção de lesões precursoras.

O adenoma constitui a lesão precursora da larga maioria dos CCRs e as características dos adenomas na colonoscopia basal de rastreio permitem definir programas de vigilância adequados após polipectomia, com impacto na sobrevida ao longo de mais de 10 anos.[7] Será então razoável pensar que se compreendermos os mecanismos moleculares subjacentes ao aparecimento dos adenomas, poderemos ser ainda mais eficazes a identificar indivíduos e janelas temporais de risco e a desenhar estratégias individualizadas de prevenção de CCR. Ou seja, para conseguir obter o controlo do CCR, será essencial compreender a sua origem.

1.2. A origem do cancro colo-rectal

1.2.1. A célula estaminal

O conceito de que nem todas as células de um cancro têm a capacidade de gerar um novo clone tumoral quando transplantadas vem já da década de 70 do século passado,[10,11] mas só vários anos mais tarde despertou a atenção da comunidade científica, com a teoria da célula estaminal de cancro.

A definição de célula estaminal implica, por um lado, a capacidade de autorrenovação e, por outro, a possibilidade de diferenciação em qualquer tipo celular de um organismo (totipotência) ou tecido (multipotência).[12] Nos humanos, a totipotência existe numa fase muito precoce da embriogénese, mas são as células estaminais com especificidade de órgão, multipotentes, que têm sido alvo de intensa investigação.

No cólon, só em 2007 foi possível identificar definitivamente células estaminais,[13] utilizando um marcador, o gene *Lgr5* (*leucine-rich-repeat-containing* G-*protein-coupled receptor* 5), identificado por Nick Barker e Hans Clevers como marcador de diferentes células estaminais no adulto.[14] O *Lgr5* é um gene alvo da via WNT que, por sua vez, codifica uma proteína de membrana que, na presença do ligando espondina-R, modula a sinalização WNT.[15]

A via WNT canónica controla a concentração citoplasmática de ß-catenina – na ausência de ativação da via, a ß-catenina é fosforilada pelo complexo de destruição [que inclui as proteínas axina, APC, GSK3 (glicogénio sintase quinase 3) e CK-1α (caseína quinase-1α)], o que leva à sua ubiquitinação e à proteólise pelo proteosoma.[15.16] Quando os ligandos específicos de ativação da via WNT se ligam ao complexo de recetores de membrana Frizzled/LPR5/6 (proteína relacionada com o recetor das lipoproteínas 5/6), dá-se a ativação da proteína *Dishevelled* do citoplasma, que se liga à axina e leva à dissociação do complexo de destruição. Assim, há acumulação de ß-catenina no citoplasma e migração para o núcleo, onde a proteína é um co ativador dos fatores de transcrição da família TCF (T-*cell factor*), levando à transcrição de vários genes alvo.[16] O *Lgr5* é um desses genes alvo. No entanto, o que vários estudos em rato e em linhas celulares têm demonstrado é que a sobre expressão de *Lgr5* vai por sua vez antagonizar a via WNT, possivelmente por interferência com o complexo de recetores de membrana.[15] Assim, o *Lgr5* parece funcionar como um regulador

negativo intrínseco da via WNT.[15] A ligação da espondina-R ao *Lgr5* parece abolir este efeito inibitório, mas o mecanismo permanece desconhecido.[15]

As células estaminais cólicas, com expressão de *Lgr5*, localizam-se na base da cripta e já tinham sido identificadas por microscopia eletrónica em 1974, sendo denominadas «*crypt base columnar cells*» (CBCC).[17] Aparentemente, as CBCC dividem-se de forma simétrica e a adoção de um destino de célula estaminal ou célula progenitora da «*transit-amplifying zone*» é um fenómeno aleatório, de «competição neutra».[18] Dados experimentais mostraram, por um lado, que a segregação dos cromossomas das células *Lgr5* durante a mitose ocorre de forma aleatória e, por outro lado, que as células filhas destas células estaminais raramente adotam destinos divergentes.[15] Portanto, a divisão de uma célula estaminal não origina uma nova célula estaminal e outra célula cometida à diferenciação – pelo contrário, cada divisão origina geralmente duas células com o mesmo destino, de novas células estaminais ou de células progenitoras da «*transit-amplifying zone*». Está também demonstrado que, constitucionalmente, as criptas evoluem para a clonalidade,[15,18] o que implica que certas linhagens se percam (quando as duas células filhas progridem para a «*transit-amplifying zone*») mesmo quando nenhuma CBCC adquire qualquer mutação que lhe confira vantagem ou desvantagem – ou seja, de forma neutra.

Os mecanismos moleculares que controlam a divisão e o destino das CBCC são ainda desconhecidos, mas podem ser dependentes de regulação externa, mantendo as dimensões relativamente constantes do compartimento de células estaminais intestinais.[18]

Um exemplo desta regulação parece ser o efeito da ingestão calórica – a restrição calórica promove a preservação e autorrenovação das CBCC, diminuindo as «*transit-amplifying cells*» e a diferenciação, efeito este mediado pelas células de Paneth.[19]

No que diz respeito ao CCR, a demonstração de que também aqui existiam células estaminais responsáveis pela autorrenovação e pela geração de toda a população tumoral foi feita em 2007 por dois grupos independentes.[20,21] O marcador de células estaminais de cancro («*cancer stem cells*» - CSC) utilizado foi o CD133, um antigénio expresso em células estaminais hematopoiéticas e epiteliais.[22] No entanto, a demonstração de que as células estaminais cólicas podem ser a origem das CSC do CCR só veio a ser feita em 2009, num artigo que demonstrou que a deleção do gene *APC* nas CBCC levava ao rápido desenvolvimento de adenomas, as lesões precursoras de CCR.[23]

Uma vez que a quimioterapia tradicionalmente utilizada para o CCR interfere essencialmente em células em divisão acelerada, é possível que as CSC sejam poupadas e que isso possa explicar as recorrências tumorais[24] – a exposição de xenografos derivados de CSC cólicas a oxaliplatina levou a uma redução do volume tumoral, mas aumentou a percentagem de células positivas para o marcador CD133.[25]

Da mesma forma, há evidência de que as CSC podem ser as únicas células tumorais capazes de formar colónias metastáticas, o que poderá depender da sua capacidade para induzir a expressão de determinadas moléculas no estroma do órgão alvo.[26] Esta «educação do

estroma» poderá ser um passo essencial para a formação do nicho que permite a iniciação metastática e a manutenção das células estaminais. [26]

As CSC surgem assim, também, como um potencial alvo terapêutico para o futuro.

1.2.2. A cripta cólica

A unidade funcional que caracteriza o epitélio do cólon é a cripta, uma zona de invaginação do epitélio pela lâmina própria. Na base da cripta residem as células estaminais, que geram células precursoras capazes de divisão rápida durante 2-3 dias na «*transit-amplifying zone*». Destas células precursoras têm depois origem todas as células da cripta, incluindo as células epiteliais diferenciadas, que migram até à superfície, onde desempenham as suas funções até à morte celular, sendo então eliminadas para o lúmen intestinal. As células precursoras, aparentemente, podem funcionar como «células estaminais de reserva», mantendo transitoriamente a capacidade de readquirir características de CBCC perante agressões ao epitélio.[27]

As criptas intestinais formam-se apenas na vida fetal tardia,[28] já depois da definição dos diferentes segmentos cólicos. A sua formação parece resultar de um fenómeno cooperativo entre células estaminais, dependente, em parte, da via WNT, existindo inicialmente mais que uma célula estaminal por cripta.[29] Este nicho de células estaminais estará sujeito a fenómenos de evolução clonal, que incluem a perda aleatória e a seleção por mutações de algumas das linhagens e que conduzem a ciclos de extinção clonal na cripta. Eventualmente, este fenómeno de sucessão no nicho resulta na perda periódica (são propostos intervalos de 2,7 a 19 anos, com uma mediana de 8,2) de todas as linhagens de células estaminais, exceto uma, mesmo quando não ocorre qualquer mutação. No entanto, determinadas mutações, nomeadamente na via WNT, poderão conferir uma vantagem seletiva à célula estaminal, que tenderá a sobreviver aos ciclos de extinção clonal e a adquirir um maior potencial para a subsequente progressão clonal.[30]

A via WNT é a principal responsável pela proliferação e manutenção das células estaminais no epitélio cólico, mas o seu nível de atividade parece ser modulado por diversos fatores, como o NF-kB, o K-RAS, sinais de stress do retículo endoplasmático e da via de sinalização NOTCH.[31]

Estes moduladores podem atuar quer de forma autócrina, quer parácrina, sendo produzidos quer pelas células de Paneth/células secretórias da base das criptas cólicas, quer pelos miofibroblastos do estroma peri-cripta.[31, 32]

No processo de competição clonal, quer a perda do *APC*, quer a ativação do *K-RAS* podem conferir uma vantagem de seleção à célula estaminal mutada.[33, 34] No caso do *K-RAS,* esta vantagem parece resultar de um aumento da taxa de divisão celular, que aumenta a probabilidade de sobrevivência na cripta da linhagem celular mutada.[33] Apenas no contexto específico das doenças inflamatórias intestinais, a perda do *p53* também pode conferir uma vantagem de seleção.[34] No caso das mutações do *K-RAS* e do *p53*, as células mutadas podem permanecer fenotipicamente indistintas, mesmo quando em expansão clonal, até adquirirem

outras mutações.[31, 34] A expansão clonal, por sua vez, parece progredir por um processo acelerado de fissão de criptas.[33]

Há evidência de que a mutação de apenas um alelo do *APC* é suficiente para conferir alguma vantagem no processo de competição clonal na cripta.[34] Um modelo experimental que procurou, com base em observações de estudos prévios, explicar o papel de uma mutação heterozigótica do *APC* na sucessão no nicho e iniciação do CRC, concluiu que esta mutação não só aumenta a divisão simétrica das células estaminais, como a desequilibra a favor da geração de novas células estaminais, *vs* células progenitoras «*transit amplifying*».[35] Por sua vez, este desequilíbrio aumenta a probabilidade de fixação de segundas mutações, somáticas.[35]

Um mecanismo molecular sugerido para este efeito de desequilíbrio a favor da geração de novas células estaminais por uma mutação monoalélica do *APC* consiste na acumulação suficiente de beta-catenina para afetar as junções aderentes, aumentando a adesão das células estaminais ao nicho.[36] De facto, foi já demonstrado que as células estaminais mais próximas do bordo do nicho têm maior probabilidade de progredir para células precursoras da «*transit amplifying zone*» enquanto as células mais perto da base têm maior probabilidade de persistir como células estaminais.[37] Foi também demonstrado, no intestino delgado, que a beta-catenina e o TCF4 medeiam o posicionamento das células ao longo do eixo cripta-vilosidade.[38]

1.3. O mosaicismo no cancro colo-rectal

1.3.1. O conceito de mosaicismo

O mosaicismo somático é definido pela presença de populações de células somáticas geneticamente distintas no mesmo organismo.[39] Um trabalho publicado em 2008, no qual foram analisados 11 a 12 tecidos de 3 homens adultos, mostrou que o mosaicismo somático para variações no número de cópias de segmentos cromossómicos (CNV) é relativamente comum nas células humanas normais. Os autores deste trabalho propuseram a hipótese de que as CNV somáticas ocorrem como parte de um processo estocástico que, na maioria dos casos, não tem consequências fenotípicas para a célula ou clones de células que afeta. No entanto, se as CNV ocorrerem em *loci* que contenham oncogenes, ou genes supressores tumorais, em determinadas células e em determinados períodos temporais, este pode ser o passo que leva ao cancro. [39]

No mesmo artigo, é descrito como num dos indivíduos estudados se identificou um *locus* com CNV em 3 tecidos diferentes, dois deles com origem na mesoderme e o outro com componentes de origem mesodérmica e ectodérmica – este achado sugeriu um mosaicismo somático com origem numa fase precoce do desenvolvimento embrionário.[39]

Da mesma forma, Steven Frank descreveu a forma como a ocorrência de uma mutação durante o desenvolvimento embrionário conduz a um mosaicismo genético somático, cujo grau varia consoante a etapa de divisão celular pós-zigótica em que a mutação surge.[40]
Um estudo realizado no epitélio da bexiga demonstrou a existência de áreas macroscópicas monoclonais, cada uma delas presumivelmente composta por descendentes da mesma célula fundadora embrionária.[41] Os resultados apontaram para a presença de 200 a 300 «retalhos» no revestimento da bexiga de uma mulher adulta, cada um deles a ocupar uma área maior do que o esperado. Se cada um destes «retalhos» provém da mesma célula estaminal embrionária, é de esperar que qualquer mutação nessa célula que predisponha à tumorigénese possa vir a desencadear o aparecimento síncrono ou metacrónico de neoplasias derivadas de células-filhas diferentes, no mesmo «retalho» geneticamente «iniciado». Esta pode ser uma explicação para os chamados «defeitos de campo» descritos para várias neoplasias epiteliais[41] e estes «retalhos» geneticamente distintos constituem uma forma de mosaicismo.
De facto, qualquer indivíduo com cancro é, por definição, um mosaico, compreendendo, pelo menos, o genoma do seu zigoto e o genoma do cancro.[42] A questão que se coloca é compreender qual a origem, celular e sub-celular, desse mosaico e em que momento surge a divergência.

1.3.2. O mosaicismo no cancro colo-rectal

No CCR, vários dados apontam para a importância do mosaicismo somático.
Na polipose adenomatosa familiar (PAF), um síndrome hereditário de CCR causado por uma mutação germinal no gene *APC*, diferentes trabalhos publicados na última década demonstraram que alguns casos atípicos são explicados por mosaicismo.[43-45] Estima-se que o gene *APC* tenha, comparativamente com outros genes responsáveis por síndromas hereditários de CCR, uma taxa elevada de mutações *de novo* (5 a $9x10^{-6}$ mutações por gâmeta por geração)[46] e a expressão fenotípica daí resultante vai depender do momento exato na embriogénese em que a mutação ocorre. Assim, uma mutação muito precoce, antes da divisão ectoderme/mesoderme/endoderme, poderá resultar num fenótipo de PAF clássica, com manifestações cólicas e extra cólicas e com risco de 50% de transmissão a cada descendente. Já mutações que ocorram mais tarde poderão cingir-se ao cólon (ou mesmo apenas a parte dele), poderão não ser detetáveis nos linfócitos do sangue periférico (se a mesoderme não tiver sido afetada), ou poderão mesmo ser transmissíveis à descendência sem que o indivíduo tenha expressão fenotípica (se a mutação afetar apenas as células germinais primordiais).[43-45] Em suma, a expressão fenotípica de uma mutação no gene *APC* depende, não só do tipo e local da mutação no gene, mas também do momento na embriogénese em que esta ocorre.
Já no CCR esporádico, não existem dados que demonstrem a existência de um mosaicismo genético somático. No entanto, vários factos apontam para essa possibilidade:

- Diferenças cancro do cólon *vs* recto: As distintas drenagens venosa e linfática do recto e do cólon podem explicar parte das diferenças encontradas na história natural, padrão de disseminação e resposta à terapêutica dos tumores nas duas localizações. No entanto, apesar da caracterização molecular realizada pela *Cancer Genome Atlas Network* não ter demonstrado diferenças significativas entre tumores do cólon e recto,[47] outros estudos demonstraram a existência de diferenças bioquímicas[48] e de expressão proteica.[49,50] Mesmo se se considerar apenas o subgrupo de tumores com instabilidade de microssatélites de alto grau (MSI-H), verifica-se que a frequência de mutações em genes alvo desta via de carcinogénese difere significativamente entre os tumores MSI-H do cólon e do recto.[51] O facto do desenvolvimento embrionário do recto ser complexo e difícil de separar do desenvolvimento do canal anal[52] pode ser a explicação para estas diferenças.

- Diferenças cancro do cólon direito/cólon esquerdo: A noção de que os carcinomas do cólon proximal têm características diferentes dos do cólon distal existe desde a década de 1980.[53] Existem diferenças epidemiológicas (idade de aparecimento, sexo mais atingido, áreas geográficas de maior incidência),[54] mas sobretudo diferenças morfológicas e moleculares claras – os adenocarcinomas de tipo mucinoso, os tumores com MSI-H e a hipermetilação de ilhas CpG (CIMP) são significativamente mais frequentes no cólon direito.[54-56] Esta dicotomia talvez possa constituir uma visão demasiado simplista, uma vez que há evidência de que determinadas características moleculares dos CCR variam gradualmente ao longo do cólon e não abruptamente quando se ultrapassa o ângulo esplénico.[57] No entanto, a visão alternativa que se propõe não é a de um cólon homogéneo, mas sim multissegmentar[58] – o que poderia equivaler a um mosaicismo somático.

- Evidência de «defeitos de campo» no cólon: No campo da carcinogénese colo-rectal associada à doença inflamatória intestinal (DII), foi demonstrada a presença de monoclonalidade em carcinomas e lesões displásicas próximas e a sua origem a partir de «retalhos» de mucosa com uma mutação fundadora comum. Em lesões distantes, por outro lado, foram identificadas mutações distintas. Este padrão de cancerização segmentar ajusta-se à ideia de lesões neoplásicas próximas com origem em células descendentes de uma única célula estaminal.[59] No caso do CCR esporádico, um trabalho mostrou a existência de defeitos de campo na mucosa cólica aparentemente normal, caracterizados por perda de expressão da metilguanina DNA metiltransferase (MGMT).[60] No entanto, não é claro se este é um evento iniciador na via de carcinogénese por instabilidade de microssatélites ou se traduz apenas a expansão clonal segmentar de outra mutação precursora ainda não identificada.[61]

Em suma, se o mosaicismo somático de base genética e com origem na embriogénese não está demonstrado no CCR esporádico, os dados disponíveis na literatura permitem colocá-lo como uma hipótese plausível.

1.4. As vias de carcinogénese colo-rectal

Em 1990, Fearon e Vogelstein publicaram um artigo fundamental para o conhecimento da carcinogénese colo-rectal. Os autores salientaram a necessidade da acumulação de várias mutações (pelo menos quatro, em oncogenes e/ou em genes supressores tumorais) para a formação de um CCR e realçaram que, embora determinadas sequências de eventos sejam mais frequentes, é esta acumulação de mutações, mais do que a sua ordem, que conduz às propriedades biológicas do tumor.[62] Neste artigo, foi ainda colocada a hipótese de que o modelo recessivo dos genes supressores tumorais de Knudson[63] possa não se verificar em todos os casos. Na sua forma canónica, a hipótese de Knudson propõe que o efeito pró-tumorigénico da perda de um gene supressor tumoral só ocorre depois da perda cumulativa dos dois alelos.[63] Contudo, há várias observações a sugerir que determinadas mutações no 1º alelo de alguns destes genes podem conferir vantagens seletivas à célula portadora, mesmo na presença de um alelo *wild-type*.[62] Finalmente, Fearon e Vogelstein admitiram que embora a maioria dos tumores tenha em comum mutações em determinados genes, mutações adicionais noutros genes ocorrem com uma frequência mais irregular, o que poderia explicar a heterogeneidade das propriedades biológicas dos tumores encontrados na prática clínica.[62]

De facto, a caracterização molecular do CCR realizada pela *Cancer Genome Atlas Network*, confirmou que, por um lado, em 93% de todos os tumores a via de sinalização WNT estava alterada, com inativação bialélica do *APC* ou mutações ativadoras do *CTNNB1* em cerca de 80% dos casos.[47] Por outro lado, verificou-se a presença de, pelo menos, dois grupos distintos de tumores, os «hipermutados» e os «não-hipermutados», com diferentes perfis de genes mutados.[47]

Os tumores «não-hipermutados», que apresentam mutação do *APC* em 81% dos casos, têm um padrão característico de CNV, expressão génica, metilação de DNA e alterações de miRNA, independentemente da sua localização anatómica.[47] Estes tumores totalizaram 84% dos casos estudados[47] e parecem corresponder ao grupo 4 da classificação proposta por Jass – tumores CIMP-negativos, com instabilidade cromossómica, maioritariamente sem instabilidade de microssatélites (MSS) e derivados de adenomas «tradicionais» (*vs* serreados).[64] Este tipo de CCR, o grupo da via de instabilidade cromossómica, corresponde não só à maioria dos tumores esporádicos,[47,64] mas também aos tumores associados à PAF e à polipose associada ao *MUTYH* (PAM).[64] O gene *APC*, cuja mutação é o passo fulcral para a maioria dos tumores que evoluem por esta via, é um exemplo de gene supressor tumoral que parece «escapar» à regra de Knudson.[63] A presença de apenas um alelo mutado deste gene parece aumentar a sobrevida da célula estaminal,[65] ou desviar a divisão da célula estaminal para a geração de duas novas células estaminais,[35] iniciando a carcinogénese.

As lesões precursoras desta via de carcinogénese, os adenomas «tradicionais» (que incluem os adenomas tubulares, vilosos e tubulo-vilosos, com displasia de baixo ou alto grau), constituem a lesão precursora de CCR mais frequente, ao surgir quer nesta via de instabilidade cromossómica, quer no contexto hereditário da via da instabilidade de microssatélites.[64] Por

outro lado, os pólipos serreados, reclassificados pela OMS em 2010 e que incluem os pólipos hiperplásicos, os adenomas serreados sésseis com ou sem displasia citológica e os adenomas serreados tradicionais – dos quais os três últimos têm potencial maligno reconhecido - constituem a lesão percursora de CCRs que evoluem pelas vias de instabilidade de microssatélites esporádica, ou pela via CIMP.[64,66]

Na via da instabilidade de microssatélites, constata-se uma divergência de mecanismos moleculares maior que na via anterior e em grande parte ainda desconhecida. Os tumores MSI-H ocorrem quer num contexto hereditário, o do Síndrome de Lynch (SL), quer no contexto esporádico.

Os CCR do SL, que ocorrem em doentes com mutação num gene de reparação do DNA (genes MMR: *MLH1*, *MSH2*, *MSH6*, *PMS2*), ou no gene *EPCAM* (cuja deleção leva à perda de expressão de MSH2),[67] são tipicamente CIMP-negativos, não apresentam mutação do *BRAF* e derivam de adenomas «tradicionais».[64] Embora estejam descritas criptas cólicas com deficiência MMR (por análise imunohistoquímica) e DNA MSI-H em mucosa cólica macroscopicamente normal de doentes com SL,[68] sugerindo que este defeito preceda a formação do adenoma, esta sequência não está demonstrada. É possível que o adenoma surja de forma idêntica à da via anterior e que só depois ocorra a deficiência MMR (através da mutação somática do segundo alelo), acelerando a progressão para CCR.[69]

Em relação aos tumores MSI-H esporádicos, a heterogeneidade aumenta, identificando-se frequentemente mutação no *BRAF*, mas também mais raramente no *KRAS*, no *CTNNB1* e/ou em diversos outros genes menos frequentemente mutados.[70] Estes tumores, que correspondem à maioria dos tumores «hipermutados» (mais frequentes no cólon direito) do *Cancer Genome Atlas Network*[47] e ao grupo 1 da classificação de Jass,[64] parecem derivar de pólipos serreados e apresentam geralmente metilação do promotor do *MLH1*, num contexto de CIMP-positivo *high*[64] - um exemplo da sobreposição das vias de carcinogénese colo-rectal.

Os tumores que evoluem predominantemente pela via CIMP vão então incluir os dois grupos restantes da classificação de Jass: o grupo 2, de tumores CIMP-positivo *high*, MSS ou MSI-L, com mutação do *BRAF* e origem em pólipos serreados; o grupo 3, de tumores CIMP-positivo *low*, MSS ou MSI-L, com mutação do *KRAS* e origem em pólipos serreados ou adenomas «tradicionais».[64] O fenótipo dos tumores desta via caracteriza-se pela hipermetilação de múltiplos genes, sabendo-se que a metilação das regiões promotoras dos genes conduz ao seu silenciamento. Está descrito o silenciamento por hipermetilação de vários genes envolvidos na carcinogénese colo-rectal, incluindo *MLH1*, *APC*, *MCC*, *MGMT*.[71] É possível que a heterogeneidade encontrada dentro da via CIMP derive de diferentes conjuntos de genes silenciados por hipermetilação. Também nesta via de carcinogénese, estudos em que se avaliou a mucosa cólica macroscopicamente normal de indivíduos com e sem CCR, identificaram padrões de metilação associados a CCR, sugerindo um defeito de campo limitado a pequenas áreas.[72]

Em suma, a divergência das várias vias de carcinogénese colo-rectal parece começar muito antes das manifestações fenotípicas, na iniciação e progressão tumoral subclínicas.

Independentemente da via de carcinogénese, no CCR são detetáveis eventos moleculares precoces mesmo em mucosa macroscopicamente normal e os dados favorecem a existência de alguma forma de mosaicismo.

2. Hipótese de trabalho

Admite-se a existência de um mosaicismo cólico de origem embrionária, em que alguns indivíduos apresentarão retalhos de mucosa cólica derivados de uma única célula estaminal embrionária com mutação num alelo de um dos genes envolvidos na carcinogénese colo-rectal.

Considerando a via de carcinogénese mais comum, a da instabilidade cromossómica, coloca-se a hipótese de que a primeira mutação, monoalélica, do gene *APC*, presente no CCR de um indivíduo esteja presente, também, noutras lesões e em retalhos de mucosa normal onde existam criptas em que uma das células estaminais provém da mesma linhagem embrionária que originou a célula estaminal tumoral. Prevê-se que a probabilidade de identificar a mutação seja maior em lesões e retalhos de mucosa próximos do tumor e, sobretudo, na mesma metade do cólon, uma vez que a formação das criptas ocorre já depois da divisão do tubo digestivo embrionário em três segmentos (*foregut*, *midgut* e *hindgut*) e o cólon direito e o cólon esquerdo provêm de segmentos distintos.

Considerando esta hipótese de trabalho, esperar-se-ia que, em indivíduos sem risco familiar para CCR, os clones derivados de células estaminais mutadas originassem adenomas dentro de uma mesma janela temporal. Se neste período estes clones fossem eliminados, através de polipectomias, a incidência de adenomas iria decair até zero. (Artigo 1) Esperar-se-ia também que, em indivíduos com CCR esporádico, os adenomas síncronos, quando presentes, fossem mais frequentes na mesma metade do cólon. (Artigo 2) Finalmente, prever-se-ia que após identificar as mutações dos dois alelos do gene *APC* presentes em CCRs de indivíduos com CCR esporádico, seria possível identificar uma mutação em comum em retalhos de mucosa cólica macroscopicamente normal do mesmo indivíduo, nomeadamente na mesma metade do cólon. (Artigo 3)

3. Material e métodos

4. Resultados

O trabalho relativo a esta tese de doutoramento desenvolveu-se em 3 fases distintas e cada uma delas culminou num artigo científico, publicado ou enviado para publicação. Assim, as secções «Material e métodos» e «Resultados» apresentam-se como parte dos respetivos artigos, que se anexam.

Artigo 1

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BRIEF ARTICLE

Adenoma incidence decreases under the effect of polypectomy

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Abstract

AIM: To investigate whether, under the influence of polypectomy, the incidence of adenoma decreases with age.

METHODS: Consecutive patients with colonic adenomas identified at index colonoscopy were retrospectively selected if they had undergone three or more complete colonoscopies, at least 24 mo apart. Patients who had any first-degree relative with colorectal cancer were excluded. Data regarding number of adenomas at each colonoscopy, their location, size and histological classification were recorded. The monthly incidence density of adenomas after the index examination was estimated for the study population, by using the person-years method. Baseline adenomas were excluded from incidence calculations but their characteristics were correlated with recurrence at follow-up, using the χ^2 test.

RESULTS: One hundred and fifty-six patients were included (109 male, mean age at index colonoscopy 56.8 ± 10.3 years), with follow-up that ranged from 48 to 232 mo. No significant correlations were observed between the number, the presence of villous component, or the size of adenomas at index colonoscopy and the presence of adenomas at subsequent colonoscopies (P = 0.49, 0.12 and 0.78, respectively). The incidence of colonic adenomas was observed to decay from 1.4% person-months at the beginning of the study to values close to 0%, at 12 years after index colonoscopy.

CONCLUSION: Our results suggest the sporadic formation of adenomas occurs within a discrete period and that, when these adenomas are removed, all neoplasia-prone clones may be extinguished.

Key words: Colorectal cancer; Colorectal adenoma; Incidence; Age; Polypectomy

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INTRODUCTION

It has long been believed that initiation of sporadic colorectal cancer (CRC) increases over time, due to toxicity or loss of fidelity of DNA replication. Therefore, it is believed that the risk of adenoma formation is a function of age[1], which justifies lifelong colonoscopy surveillance. However, current evidence has started to challenge these perceptions.

The data that have suggested that incidence of adenoma increases with age were mostly collected from autopsy studies[2,3]. Although relevant, these studies are prone to bias because the age-specific incidence rates do not adequately represent the time trend for newly formed lesions after removal of index lesions. Moreover, the rather important information of family aggregation of CRC was lacking in all of them.

More recent studies, based on surveillance colonoscopies[4-6], have been mainly limited by short follow-up (3-5 years), which may lead to the inclusion, as new adenomas, of polyps that were missed at a previous examination. These time intervals are also likely to be too short to predict the lifelong dynamics of adenoma formation.

The notion that the formation of colon adenomatous polyps peaks at a certain age and then rapidly declines was first presented in 1975 by Henry *et al*[7]. He stated that: "some undefined stimulus to continuous polyp formation persists for up to 4 years in about one-third of patients who develop a colonic polyp. Thereafter, either the stimulus to neoplasia is no longer present, or the colonic mucosa adapts so that polyp formation does not persist." We propose that this undefined stimulus corresponds to the presence of a limited number of mutated clones that are scattered in the colonic epithelium and evolve through cumulative critical gene events selected over several decades. This sequence of events ends at a specific time window, in which most adenomas become endoscopically detectable and after which its incidence declines.

A study in Germany[8] has shown that individuals with negative findings at colonoscopy had a reduced risk of CRC for at least 20 years. Furthermore, when the examination was performed at 55-64 years of age and older, the risk of CRC was even lower. Additionally, according to the latest Guidelines for Colonoscopy Surveillance after Polypectomy issued by the American Gastroenterological Association (AGA)[9], age is not considered to be a reliable predictor of subsequent advanced adenomas.

Taking these data into account and considering the possibility of only a limited time window for sporadic adenoma expression, we aimed to study the temporal trend for adenoma formation, in a standard-risk population under colonoscopic surveillance. According to our hypothesis, which stated that a discrete number of mutated clones were scattered in the colon, we expected the adenoma incidence to decrease under the effect of polypectomy.

MATERIALS AND METHODS

Patients and procedures

Consecutive patients from three Portuguese hospitals (a tertiary oncology center, a tertiary general hospital and a regional hospital) with colonic adenomas who were identified at an index colonoscopy were retrospectively reviewed. These patients were included if they had undergone three or more complete colonoscopies that were at least 24 mo apart. The index colonoscopies were performed between 1978 and 2000, for screening or diagnostic purposes. The subsequent colonoscopies were performed according to the assistant physicians' choice, based on surveillance guidelines that changed during the time of the study (data were collected until 2007). Only colonoscopies reaching the cecum, with adequate bowel preparation and complete removal of all of the identified polyps were considered for inclusion in the present study.

The patient files were reviewed, and the patients were excluded if they had a previous history of CRC or adenomas, inflammatory bowel disease, hereditary non-polyposis CRC, familial adenomatous polyposis syndrome, a family history of CRC in any first-degree relative, or CRC at the index colonoscopy.

The location and size of all of the polyps had been recorded and the specimens had been sent for pathological evaluation and were classified according to the criteria of the World Health Organization[10]. All of the colonoscopies were performed by certified gastroenterologists, and sedation using intravenous midazolam (with or without pethidine association) or intravenous propofol (performed by an anesthesiologist) was administered on a case-to-case basis. The bowel preparation methods varied among the centers and over time, but were based on oral solutions that contained polyethylene glycol or senna.

The endoscopic and pathological reports were reviewed by the authors, for the results of the index colonoscopy, and of each colonoscopy reported thereafter. The left colon was defined as the splenic flexure and the segment distal to it.

The baseline adenoma characteristics were recorded and correlated with recurrence of adenomas at follow-up. However, these adenomas were specifically excluded from incidence ratio evaluation, because the time frame for their formation was unknown.

Data analysis

The data regarding number of adenomas observed at each colonoscopy, along with their location, size and

Table 1 Outcomes of first four colonoscopies

Outcomes	Mean	SD
Index colonoscopy: 156 patients studied		
Adenomas	1.68	1.01
Tubular adenomas	1.49	1.13
Tubulo-villous/villous adenomas	0.14/0.05	0.35/0.22
Size of the larger adenoma (mm)	15.51	10.36
Adenomas in the left colon/remaining colon	1.34/0.35	0.93/0.69
Second colonoscopy: 156 patients studied (41.6 ± 21.0 mo since the first)		
Adenomas	0.54	0.86
Tubular adenomas	0.51	0.85
Tubulo-villous/villous adenomas	0.02/0.01	0.14/0.11
Size of the larger adenoma (mm)	8.02	7.39
Adenomas in the left colon/remaining colon	0.36/0.17	0.67/0.49
Third colonoscopy: 156 patients studied (83.7 ± 27.7 mo since the first)		
Adenomas	0.47	0.92
Tubular adenomas	0.46	0.92
Tubulo-villous/villous adenomas	0.02/0.0	0.18/0.0
Size of the larger adenoma (mm)	6.19	4.22
Adenomas in the left colon/remaining colon	0.21/0.26	0.47/0.71
Fourth colonoscopy: 44 patients studied (116.9 ± 34.1 mo since the first)		
Adenomas	0.32	0.74
Tubular adenomas	0.32	0.74
Tubulo-villous/villous adenomas	0.0/0.0	0.0/0.0
Size of the larger adenoma (mm)	5.78	1.92
Adenomas in the left colon/remaining colon	0.16/0.16	0.43/0.48

histological classification were recorded. The analysis of adenoma incidence over time was based on the person-years method. We assumed that the incidence rate of adenomas was constant between two consecutive colonoscopies in each individual and, in that time interval, the monthly incidence of adenomas in each patient was estimated by dividing the number of adenomas found at colonoscopy by the number of months that had elapsed since the previous examination. The incidence of adenomas in a given month after the index colonoscopy was determined for the entire sample by summing the incidence for that month across all of the patients that had been observed up to that time. The monthly incidence density of adenomas was then obtained by dividing the estimated incidence in that month by the population that was still at risk.

The statistical analysis was performed using Excel XP (Microsoft Inc) and Stata 10.0 (Stata Corporation, College Station, TX, United States). The baseline adenoma characteristics were correlated with recurrence at follow-up using the χ^2 test.

Ethics

The present study was a retrospective observational study, in which no experimental intervention was used, and all of the data were kept anonymous. Therefore according to the local regulations, no approval by the Ethics Committee was necessary. All of the patients signed an informed consent document before each endoscopic examination.

RESULTS

The present study included a total of 156 patients (109 male and 47 female). The mean age at the time of the index colonoscopy was 56.8 ± 10.3 years. No procedural complications were recorded. All 156 patients underwent three colonoscopies and 44 of them underwent a fourth examination. The outcomes of these colonoscopies are summarized in Table 1. The index colonoscopy was performed for screening in 31 patients and for diagnostic purposes in the remaining patients (and the symptoms were considered to be unrelated to the adenomas in the majority of cases).

Table 2 Correlation between baseline adenoma characteristics and presence of adenomas in the second colonoscopy

Index colonoscopy	Second colonoscopy		χ^2 test
	0 adenomas (*n*)	⩾ 1 adenoma (*n*)	
> 1 adenoma	39	28	$P = 0.24$
⩾ 3 adenomas	14	10	$P = 0.57$
⩾ 1 TV adenoma	8	14	$P = 0.004$
⩾ 1 villous adenoma	5	3	$P = 0.95$
⩾ 1 TV or V adenoma	13	17	$P = 0.01$
⩾ 1 adenoma > 1 cm	67	42	$P = 0.43$

TV: Tubulo-villous; V: Villous.

The total number of adenomas and the numbers of each adenoma subtype (according to the histology or location) declined over the course of the four colonoscopies. Additionally, 12 patients underwent a fifth examination (162.3 ± 32.7 mo after the first), and two of these had a sixth examination (211.5 ± 20.5 mo after the first). No adenomas were found in any of these last examinations.

Of the initial 156 patients, who underwent three colonoscopies, 107 presently have scheduled colonoscopies, in agreement with the latest guidelines for surveillance after polypectomy; 27 patients have been released from follow-up due to advanced age or significant comorbidity; two patients have died of unrelated causes; and 20 have been lost from follow-up.

There was no significant correlation between the number of adenomas at the index colonoscopy and the presence or absence of adenomas at the second or all subsequent colonoscopies ($P = 0.68$ for the second colonoscopy, $P = 0.49$ for all subsequent colonoscopies). The presence of three or more adenomas at the index colonoscopy did not correlate with the presence of adenomas at the subsequent colonoscopies ($P = 0.57$ for the second, $P = 0.21$ for all subsequent colonoscopies), nor did the presence of adenomas > 1 cm at the index colonoscopy ($P = 0.43$ for the second colonoscopy, $P = 0.78$ for all subsequent colonoscopies). The presence of adenomas with a villous component at the index colonoscopy correlated with the presence of adenomas at the second colonoscopy ($P = 0.01$), but there was no significant correlation when all of the subsequent colonoscopies were considered together ($P = 0.12$) (Tables 2 and 3).

The presence of adenomas of the left colon at the index colonoscopy did not predict recurrence in the same segment in the second or all subsequent colonoscopies, and the same was true for the right colon (data not shown).

The incidence of colonic adenomas was found to

Table 3 Correlation between baseline adenoma characteristics and presence of adenomas in all subsequent colonoscopies

Index colonoscopy	Subsequent colonoscopies		χ^2 test
	0 adenomas (*n*)	⩾ 1 adenoma (*n*)	
> 1 adenoma	31	36	*P* = 0.5
⩾ 3 adenomas	9	15	*P* = 0.2
⩾ 1 TV adenoma	7	15	*P* = 0.08
⩾ 1 villous adenoma	4	4	*P* = 0.97
⩾ 1 TV or V adenoma	11	19	*P* = 0.12
⩾ 1 adenoma > 1 cm	53	56	*P* = 0.78

TV: Tubulo-villous; V: Villous.

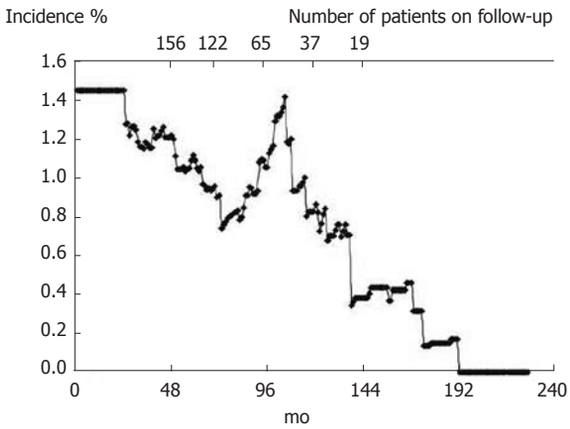

Figure 1 Variation of colonic adenomas monthly incidence (person-month percentage) with time, since the index colonoscopy.

decline from 1.4% person-months, at the beginning of the study, to values close to 0%, 12 years after the index examination (Figure 1).

Eight years after the index colonoscopy (with 65 patients evaluated), a peak in the incidence of adenomas was observed, that approached the baseline values, which was then followed by a steady decline until the end of follow-up (with 37 patients evaluated at 10 years and 19 patients evaluated at 12 years).

There were no reports of flat lesions of the colon in any of these examinations, and there were no colorectal adenocarcinomas reported in these patients during the study period.

DISCUSSION

To explain the predominant CRC expression in the sixth and seventh decades of life, a necessary sequence of 4-7 known mutations fits a model of a stable mutation clock that ends in full-blown neoplasia at consistent time-intervals. The predominant molecular pathway responsible for CRC begins with the selection of cells with an *APC* gene loss or a β-catenin mutation, which is followed by the cumulative selection of subsequent critical events in other genes. Estimations of the time taken to acquire such a sequence of mutations suggest that full-blown neoplasia can take several decades to occur. According to recent estimations, to obtain the necessary sequence of mutations, the first event may need to occur at an early age[11-13]; most likely during the exponential phase of embryonic development, when *APC* is well known to play a key role[14].

In 2007, human colon cancer stem cells were identified by two separate research groups[15,16]. More recently, the location of normal colon stem cells, at the crypt base, has also been demonstrated[17], and these cells seem to be the origin of colon cancer stem cells[18]. It has been proposed that mutations in stem cells are much more likely to occur during the exponential phase of early growth, as opposed to later in life[19].

Accordingly, the results of the present paper strongly suggest that the sporadic formation of adenomas occurs during a limited time period and, when these adenomas are removed, virtually all of the neoplasia-prone clones may be extinguished. If this model is shown to be true, then the concept of a field carcinogenic defect, which progresses with continuous adverse environmental exposure and/or failure of the tight controls that assure DNA replication fidelity, should be replaced by a concept of limited colonic mosaicism. When the endoscopically visible neoplastic expression of this limited mosaicism is removed, the putative carcinogenic impulse no longer compromises colonic epithelia homeostasis.

Our study was limited mainly by its retrospective nature and by the small number of patients reaching longer follow-up. However, this sample was probably representative of the population at risk for sporadic CRC, because it included both symptomatic and asymptomatic individuals who had no relevant family histories, who underwent initial colonoscopy mostly during the sixth decade of life, and who were followed for at least 4 years.

In contrast to traditional beliefs, we did not observe a correlation between baseline adenoma characteristics and the risk of recurrence at follow-up. Although the AGA still takes baseline predictors of future adenomas or cancer into consideration, in their latest Guidelines for Colonoscopy Surveillance After Polypectomy[9], several limitations of the available evidence have been raised. van Stolk *et al*[20] have reported a study in which the number of adenomas at first colonoscopy was a significant predictor of having recurrent adenomas; however, these authors noted that missed polyps were a possible explanation for this relationship. Furthermore, that previous study only included 4 years of follow-up, and the presence of a family history of CRC was not taken into account at all. In another study, by Martínez *et al*[21], multiple adenomas at baseline, large adenomas (> 1 cm) or adenomas in the proximal colon were predictors of recurrence. However, the maximum follow-up of that study was 2 years, and the baseline colonoscopy was not the first examination for several of the patients. In addition, and although this relationship was not statistically significant, a family history of CRC in first-degree relatives was associated with a higher risk of recurrence in the study population. The initial National Polyp Study also included a follow-up of only 3 years, and investigated the incidence of colorec-

tal cancer but not adenoma[2,22]. The authors included patients regardless of their family history (other than established genetic syndromes)[2] and they admitted that, as a result of the short follow-up, three of the diagnosed cancers may have been missed polyps[23]. A more recent study, by Martinez *et al*[24], has revealed that the age of the patient and the number and size of prior adenomas were associated with the risk of advanced colorectal neoplasia after polypectomy. However, that study was also limited by a median follow-up of only 4 years and a maximum follow-up of < 6 years[24]. Our study had the advantages of a minimum follow-up of 4 years and exclusion of patients with any family history of CRC in first-degree relatives. This might explain why we did not find that baseline adenoma characteristics were predictive of recurrence, and why we were able to show a decreasing incidence of adenomas with time, after an age peak and under the influence of polypectomy.

At Digestive Disease Week 2007, Zauber *et al* presented new data regarding the National Polyp Study population indicating that, after a mean follow-up of 14 years, CRC mortality was markedly reduced in all of the patients with adenomas at baseline, when compared to the general population. This was observed even when patients who refused follow-up were considered in the analysis. This finding supports the hypothesis that the major benefit is derived from the first colonoscopy and also brings into question the notion of increasing recurrence of adenomas over time.

With regard to the unexpected second peak that was observed for the incidence of adenomas in our study, after 8 years, we may speculate whether this peak is related to the type of the second hit in the *APC* gene. It has been reported that, when the first hit happens close to codon 1300, the second hit is most likely loss of heterozygosis, a faster and more efficient mutation process. In all other cases, point mutation is the most common mechanism for the second hit, thus driving a more sluggish development[25]. This confers different selective advantages to each colonic adenoma, according to the specific first hit-second hit combination and may explain why some adenomas appear earlier and why others are only apparent towards the end of the adenoma time window. We acknowledge that, during a limited period of embryonic development, a small number of stem cells may acquire different first mutations, and that these mutations are expressed along incidence waves during a restricted time window.

The results of the present study reinforce the importance of colonoscopy with polypectomy during the fifth to sixth decades of life, and the feasibility of increasing the time for re-examination after a normal examination. Furthermore, our study indirectly supports the concept of a relatively stable mutation clock, which is possibly initiated during the developmental phase of embryogenesis.

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COMMENTS

Background

Colorectal cancer (CRC) and adenomas are thought to have an increasing incidence with age, and this has led to almost lifelong surveillance colonoscopy. This view has recently started to be challenged.

Research frontiers

APC is a crucial gene in colorectal carcinogenesis. If the first hit on this gene occurs in a colonic stem cell precursor early in life, it could lead to only a few colon crypts that are prone to originate adenoma and cancer, in a restricted time window. If these clones were removed, by polypectomy, the potential for colon cancer could be erradicated in that individual.

Innovations and breakthroughs

Colon cancer stem cells have recently been identified, apparently originating from colonic stem cells, at the crypt base. Stem cells seem more prone to mutation during embryogenesis than later in life. Reports have shown that a single colonoscopy with polypectomy reduces CRC incidence. The study shows that, under the influence of polypectomy, adenoma incidence decreases with age.

Applications

If the study results are confirmed, surveillance colonoscopy intervals may safely be lengthened, and some people may even be released from surveillance after a few examinations.

Terminology

APC acts as a tumor suppressor gene by regulating the intranuclear concentration of β-catenin, a protein involved in the transcription of genes that promote proliferation. These genes and proteins are part of the Wnt pathway, which is involved in > 80% of sporadic CRC.

Peer review

The authors do a nice job to expand on a current line of questioning the need for continuing surveillance and indirectly questioning the concept of age appropriateness for surveillance as well as cost-advantageous practice. This is a very relevant study and well written.

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Artigo 2

The co-localization of carcinomas and adenomas favors a regional field defect in the colon: an observational study

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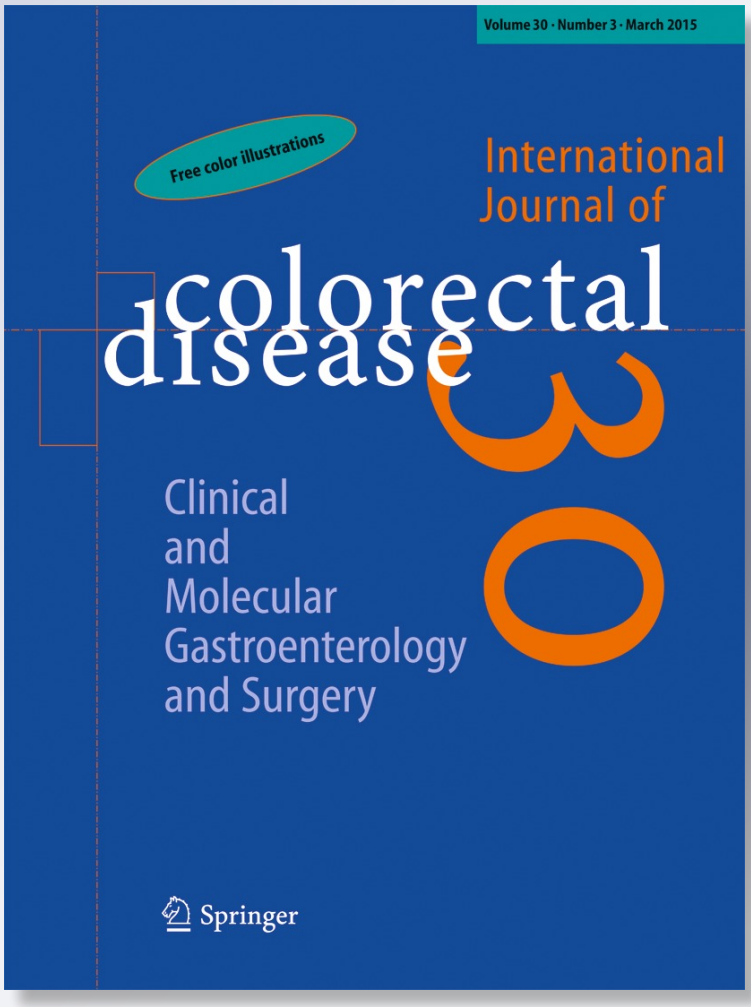

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ORIGINAL ARTICLE

The co-localization of carcinomas and adenomas favors a regional field defect in the colon: an observational study

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Abstract
Purpose Finding common genetic alterations in colorectal cancers (CRCs) and peri-tumoral mucosa first led to the notion of colonic field defects. The hypothesis of a genetically determined mosaicism would explain these defects and would make the co-localization of tumors likely. Our purpose was to indirectly test this hypothesis by searching for a possible correlation between the location of colorectal cancers and adenomas
Methods This is a retrospective observational study. Patients operated for colorectal cancers at an oncological hospital, who had a full colonoscopy performed in the two peri-operative years, were surveyed. Sex, age, familial risk of cancer, tumor and adenoma locations, and the presence of adenomas larger than 1 cm, with villous component or high-grade dysplasia were recorded. Statistics: *T* test, chi-square, exact, logistic regression (SPSS18®).
Results This study included 224 patients (57 % male, mean age 67.6 years), 45 % of which had synchronous adenomas. There was a significant correlation between cancer location and location of all adenomas (p=0.01) and of adenomas larger than 1 cm (p=0.01). Adenomas of the right colon were more frequent in patients with right colon cancer (p=0.008), and the same was true on the left colon (p=0.002).
Conclusions The strong correlation between the locations of CRC and synchronous adenomas, namely risk adenomas, may point to a common early defect. It does also suggest that hemicolectomy may always be the surgery of choice for colon cancer.

Keywords Colorectal cancer · Synchronous colorectal adenomas

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Introduction

In the field of colorectal carcinogenesis, the notion of a field defect is still controversial. Genetic and epigenetic changes common to the cancer were described in macroscopically normal colonic mucosa of colorectal cancer (CRC) patients, first in ulcerative colitis patients [1] and later in standard-risk sporadic CRC patients [2]. This led to the extrapolation to the colon of the field defect theory, originated in cancer models where there is a common exposure to environmental toxicities like otorhinolaryngological cancers. In the colon, however, the notion of a structural genetic or epigenetic difference between colonic segments, namely between the right and left colon [3], has been integrated in the theory.

In fact, studies show that synchronous sporadic CRCs are more common in the same colonic half and epigenetic changes are only concordant between synchronous cancers when they occur in the same side of the colon [4]. Data regarding synchronous adenomas are sparse.

Our goal was to indirectly test the hypothesis of a colonic field defect by studying the correlation of CRCs location with the presence and location of synchronous adenomas, namely risk adenomas.

Materials and methods

Population and procedures

Clinical files of all CRC patients submitted to surgery at Instituto Português de Oncologia de Lisboa, Francisco Gentil (IPOLFG), EPE between November 2006 and June 2010 were analyzed. Patients were included if they had been submitted, in the same institution, to one or more full colonoscopies with adequate bowel preparation in the two peri-operative years. Only colonoscopies in which all polyps had been removed and sent for pathology exam were considered. Patients were only included if there were full data regarding polyp size, location, and classification according to the World Health Organization (WHO) [5].

Patients were excluded if they had previous history of colorectal resection surgery, CRC, or adenomas, inflammatory bowel disease, or family history of hereditary CRC syndromes [familial adenomatous polyposis (FAP), *MUTYH*-associated polyposis, Lynch syndrome (LS), Peutz-Jeghers syndrome or juvenile polyposis, with a genetic-based diagnosis, FAP diagnosed by the presence of more than a hundred adenomas, attenuated FAP by Nielsen et al. [6] criteria, Amsterdam II criteria for LS [7] or serrated polyposis by WHO [8] criteria]. With clinical file data, patients were stratified according to familial risk for CRC as low risk (one first-degree relative with CRC above 60 years old), moderate (one first-degree relative with CRC below 60 years old), or high risk (above moderate, but not fulfilling hereditary syndromes criteria). Patients with no family history of CRC were classified as standard risk.

All colonoscopies were performed by a certified gastroenterologist and sedation by midazolam (with or without meperidine) or propofol (under anesthesiologist supervision) was given according to clinical decision. Bowel preparation was based in polyethyleneglycol or sodium picosulfate solutions. All endoscopic and pathology reports were reviewed by study investigators.

The right colon was defined as the colon proximal to the splenic flexure, the left colon was defined as the splenic flexure and the colon distal to it, and the rectum was defined as the most distal 15 cm, measured from the anal verge.

In patients with synchronous CRCs, the one with the largest dimensions (defined by endoscopy) was considered to be the index lesion.

High-risk adenomas were defined as those larger than 1 cm and/or with villous component and/or with high-grade dysplasia.

Data analysis

Data were collected regarding the following: patients' sex and age, familial risk of CRC, cancer and adenomas' location, presence and location of adenomas larger than 1 cm, with villous component and with high-grade dysplasia.

Statistic analysis was done using SPSS 18, for Student's *T* test, chi-square test, Fisher's exact test, and logistic regression analysis (Enter method).

Results

Study population

IPOLFG, EPE is a tertiary oncological center with a Hereditary Colorectal Cancer Clinic and some colorectal cancer patients are only referred to the institution for treatment, after a diagnosis done elsewhere. During the study period, 696 patients were submitted to surgery for CRC at IPOLFG, EPE and 244 of these fulfilled the study inclusion criteria. Thirty two patients were excluded due to previous colorectal resection surgery, 30 patients were excluded due to a familial history fulfilling criteria for hereditary CRC syndromes, and the remaining 390 patients could not be included because the peri-operative colonoscopies were performed at other institutions.

The mean patient's age at surgery was 67.6±9.8 years (37–90 years) and 140 patients (57.4 %) were male.

Synchronous lesions prevalence

Synchronous CRCs were found in 7 (2.9 %) patients and synchronous adenomas were found in 110 (45.1 %) patients. For patients with synchronous CRCs, the location of the index lesion was considered for further analysis. The presence of synchronous adenomas or CRC was independent of index cancer location ($p=0.66$; $p=0.90$, respectively; Table 1).

Familial risk for CRC

Familial risk for CRC was known in 199 of the 244 patients (82 %), from which 166 (83 %) were standard-risk patients.

Table 1 Correlation of cancer location with the presence of any synchronous adenomas or cancers

		Right colon cancer	Left colon cancer	Rectal cancer	Chi-square/exact tests
Synchronous adenomas	Absent	37	45	52	p=0.66
	Present	27	43	40	
Synchronous cancer	Absent	62	85	90	p=0.90
	Present	2	3	2	

Familial risk of CRC was low in 20 patients, moderate in 7, and high in 6 patients. In these 199 patients, there were no significant correlations between familial risk stratification and the presence of synchronous cancers (p=0.60) or adenomas (p=0.61), nor with the location of index cancers (p=0.33) or synchronous adenomas (p=0.90).

Synchronous cancers characteristics

From the seven patients with synchronous CRCs, one had three CRCs in the left colon and the remaining patients had two CRCs each.

From the six patients with two CRCs, three had synchronous rectal and left colon cancers, two had synchronous left and right colon cancers, and one had synchronous rectal and right colon cancers.

Synchronous adenomas characteristics

When the 110 patients with synchronous adenomas were considered, these synchronous adenomas' location was significantly correlated to index CRC location (p=0.01; Table 2).

In these patients, having a right colon cancer increased the likelihood of having adenomas in the right colon (p=0.008; Table 3) and the same was true for the left colon (p=0.002; Table 3), but not for the rectum (p=0.61; Table 3).

Considering this result, we chose to exclude rectal cancer patients and analyze further the subset of patients with right or left colon cancer and synchronous adenomas. This subset comprised 70 patients (43 left colon cancers, 27 right colon cancers).

From these 70 patients with synchronous adenomas, there were 47 patients with at least one high-risk adenoma. Forty six patients had at least one synchronous adenoma larger than 1 cm, 16 patients had at least one tubulovillous or villous adenoma and 20 patients had at least one high-grade dysplasia adenoma.

In these 47 patients with left or right colon cancer and synchronous high-risk adenomas, CRC location was significantly correlated to the location of adenomas larger than 1 cm (p=0.01; Table 4), which were more common in the same colonic half. No correlation was found with the location of adenomas with villous component (p=0.64; Table 4) or high-grade dysplasia (p=0.53; Table 4).

Multivariate analysis

For the logistic regression analysis, all 244 patients were considered. Included variables were the following: patients' sex and age, CRC location, total number of adenomas, and number of adenomas larger than 1 cm. Right colon synchronous adenomas were significantly more likely when there was a right colon cancer (p=0.03) and when there was a higher total number of adenomas (p<0.001). The presence of left colon synchronous adenomas correlated significantly only with CRC location (p=0.007).

Discussion

In the latest years, evidence has been gathering for the stem cell origin of CRC [9–11]. As Steven Frank [12] described, the path of every stem cell-derived lineage begins in the zygote and goes through several precursor cells. If a mutation occurs during embryonic development, the number of affected stem cells in the adult colon will depend on the exact point of this sequence in which it happens. Mutations may occur after the embryonic division of the right and left colon, which precedes colonic crypts formation. This kind of event might explain the results found by Nosho et al. [4], who described

Table 2 Correlation of cancer location with the location of synchronous adenomas

		Right colon cancer	Left colon cancer	Rectal cancer	Chi-square/exact tests
Synchronous adenomas location	Right colon	15	7	12	p=0.01
	Left colon	3	20	15	
	Rectum	2	3	2	
	>1 location	7	13	11	

Table 3 Correlation of cancer location with the presence of synchronous adenomas in each colonic half and in the rectum

		Right colon cancer	Left colon cancer	Rectal cancer	Chi-square/exact tests
Right colon adenomas	Absent	5	24	18	p=0.008
	Present	22	19	22	
Left colon adenomas	Absent	18	11	13	p=0.002
	Present	9	32	27	
Rectal adenomas	Absent	22	36	36	p=0.61
	Present	5	7	4	

similar epigenetic changes found only in synchronous CRCs of the same colonic half, with non-affected mucosa between them.

Similarly, our study found a statistically significant correlation between the colonic half location of carcinomas and synchronous adenomas, namely those larger than 1 cm. The fact that we could not find the same correlation for other risk adenomas' characteristics, namely villous component and high-grade dysplasia, may simply be due to the low numbers of patients with these types of lesions. The low number of patients with synchronous CRCs in our series did not allow for the establishment of correlations in this subset, but it is noteworthy that in the only patient with three synchronous CRCs, they were all in the same colonic half.

The co-localization of carcinomas and synchronous adenomas found in our study might be explained by a segmental colonic field defect. The existence of an environmental genotoxic pressure affecting only some colonic segments has never been demonstrated. An alternative explanation is that of a genetic or epigenetic basis for the field defect, leading to the existence of a colonic mosaicism. The fact that the right and left colon have distinct embryonic origins (midgut and hindgut, respectively) contributes to the hypothesis of a colonic mosaicism originated at that period of development.

It is now clear that there are several pathways of colorectal carcinogenesis and their predominance differs between the right and left colon. Even when the most common pathway (Wnt/β-catenin pathway) is considered, there are differences between the degree of activation needed for carcinogenesis in each colonic half [13]. There is no explanation yet for these differences but it seems plausible that it goes back to embryogenesis and to this proposed mosaicism.

If the right and left colon embryonic origins are clearly established, the rectal development is more complex and hard to separate from the anal canal embryonic development [14]. Therefore, an early origin of a colonic mosaicism might also explain the results we found for the rectum, where, in this study, we could not find the correlation between carcinoma's and adenoma's location found in the colon.

Our study's main limitation was its retrospective nature, which made us exclude all patients submitted to colonoscopy outside our institution for whom we could not guarantee the standardization of endoscopic and pathological reports. This retrospective nature also explains why familial risk was unknown in 18 % of patients, but the fact that in the remaining patients, standard risk was found in more than 80 % shows our population can be considered representative of the sporadic CRC population.

Going back to a clinical point of view, our results reinforce the need to think of CRC as a manifestation of a possibly larger defect, whatever its origin. In this study, 45 % of colorectal cancer patients were found to have synchronous

Table 4 Correlation of synchronous risk adenomas location with colon cancer location

		Right colon cancer	Left colon cancer	Chi-square/exact tests
>1-cmadenomas' location	Right colon	11	7	p=0.01
	Left colon	2	13	
	Rectum	2	3	
	>1 location	1	7	
Villous adenomas' location	Right colon	4	4	p=0.64
	Left colon	1	3	
	Rectum	1	1	
	>1 location	0	2	
High-grade dysplasia adenomas' location	Right colon	6	6	p=0.53
	Left colon	1	5	
	Rectum	0	1	
	>1 location	0	1	

adenomas and 2.9 % had synchronous cancers. We therefore stress the need for at least one full peri-operative colonoscopy, given the high likelihood of identifying synchronous lesions. Our data also point to the need to consider the recommendation proposed by Japanese authors to abandon segmental colonic resections, opting only for right or left hemicolectomy [15].

Ethical statements

This was a retrospective observational study with no experimental intervention or financial support, in which data were kept anonymous and the Helsinki Declaration was respected, therefore, it did not require institutional board approval. In accordance with the institution's practice, all patients gave their informed consent for each endoscopic exam or surgical procedure.

Conflicts of interest Isadora Rosa received consultancy fees from MSD®, AbbVie®, and Dr. Falk Pharma®. She is the lead investigator in an ongoing project for which her institution received a grant from MSD®. She received support for travel/accommodations/meeting expenses from MSD®, AbbVie®, Dr. Falk Pharma® and Hospira®. All of these situations occurred outside the submitted work. The remaining authors have no potential conflicts of interest to disclosure. The study received no financial support.

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Sporadic colorectal cancer: Studying ways to an end

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Abstract

Introduction: Although colorectal cancer (CRC) has often been regarded as a single entity, different pathways may lead to macroscopically similar cancers. These pathways may evolve into a patchy colonic field defect that we aimed to study in consecutive CRC patients.

Methods: In a single-center, observational, prospective study, consecutive CRC patients were included if surgery and a perioperative colonoscopy were planned. Personal and familial history data were collected. Tumors were studied for microsatellite instability (MSI) status, DNA repair protein expression (DRPE) and presence of *BRAF* and/or *APC* mutations. Macroscopically normal mucosa samples were tested for *APC* mutations. Presence and location of synchronous and metachronous adenomas and patient follow-up were analyzed. The association of two categorical variables was tested through the Fisher's exact test (SPSS 19).

Results: Twenty-four patients (12 male, mean age 69 years) were studied. High-grade MSI (MSI-H) was found in eight tumors–these were significantly more common in the right colon ($p = 0.047$) and more likely to have an altered DRPE ($p = 0.007$). *BRAF* mutation was found in two of six tested MSI-H tumors. *APC* gene mutations were found in nine of 16 non-MSI-H tumors and absent in normal mucosa samples. There was a nonsignificant co-localization of CRC and synchronous adenomas and a significant co-localization ($p = 0.05$) of synchronous and metachronous adenomas.

Discussion: Sporadic CRCs evolve through distinct pathways, evidenced only by pathological and molecular analysis, but clinically relevant both for patients and their families. In non-MSI-H tumors, the expected *APC* gene mutations were not detected by the most commonly used techniques in a high number of cases. More studies are needed to fully characterize these tumors and to search for common early events in normal mucosa patches, which might explain the indirect evidence found here for a field defect in the colon.

Keywords

Colorectal, cancer, field defect, APC, pathways

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Introduction

It has been proposed for several decades that most cancers start from a single cell and tumor progression occurs through genetic variability in the original clone.[1] The main molecular route to colorectal cancer (CRC), the chromosomal instability pathway, starts with adenomatous polyposis coli (*APC*) gene loss or beta-catenin mutations, followed by the accumulation of critical events in other genes. The time frame estimates for this sequence of mutations to occur point to the occurrence of the first event at an early age,[2–4] probably during the exponential period of embryonic development, in which *APC* has an established determinant role.[5] CRC stem cells have been identified in humans,[6,7] and it has been argued that stem cell mutations occur

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much more frequently in the early, exponential stage of development than in adult life.[8]

The location of the epithelial colonic stem cells at crypt base was also recently demonstrated[9] and these cells seem to be the origin of CRC.[10] Intestinal crypts formation happens only at late fetal life,[11] when colonic segments are already defined, and all colonic epithelial cells arise from the pluripotent stem cells located at the crypts. Crypt development seems to emerge from a stem cells' cooperative phenomenon, partly dependent on the Wnt pathway, and each crypt seems to contain more than one stem cell.[12] This stem cell niche appears to be the site of a clonal evolution phenomenon, including the random loss and selection of some lineages, leading to clonal extinction cycles in the crypt. Eventually, this niche succession phenomenon results in the periodic (two seven- to-19-year intervals are proposed, with a median of 8.2) loss of all but one stem cell lineage. A few mutations, namely in the Wnt pathway, may give the stem cell a selective advantage, allowing it to survive the clonal extinction cycles, with great potential for clonal progression.[13] Recent experimental data point to a relatively low mutation rate in CRC almost to its late stages, quite similar to the division and mutation rates at normal mucosa. This might be explained by an early beginning of cancer genealogy, close to conception, followed by decades of evolution in normal-appearing mucosa.[13,14]

In the clonal competition process, it appears that either *APC* loss or *K-RAS* activation may lead to a selection advantage for the mutated stem cell.[15,16] There is evidence that even a single allele mutation in the *APC* gene is enough to confer some advantage in the crypt clonal competition process.[16] Mutated cells may remain phenotypically undetectable, even while in clonal expansion, until they acquire other mutations.[16,17] Although CRC is regarded as a single entity, different first events may evolve through distinct pathways before they lead to macroscopically similar cancers.

The occurrence of a mutation during embryonic development leads to a genetic mosaicism, the degree of which changes according to the post-zygotic cell division stage in which the mutation occurs.[18] Therefore, the occurrence of a monoallelic gene mutation in a stem cell during embryonic development would lead to a somatic colonic mosaicism, in which the mutation would be identified in discrete colonic mucosa patches. These patches would correspond to the most likely segments for the development of adenomas, and subsequent evolution to cancer.

We therefore hypothesize that, when the most common pathway to CRC is considered, the first, monoallelic, mutation of the *APC* gene present in an individual's CRC is also present in other colonic lesions and normal mucosa patches where some crypt stem cells derive from the same embryonic lineage that gave rise to the CRC stem cell. We predict that the probability of identifying the mutation will be greater in lesions and patches closer to the tumor.

Therefore, we aimed to characterize the pathways leading to sporadic CRC in a random sample and to search for mucosal patches with a mutated *APC* allele in those patients with a chromosomal instability pathway cancer.

Sporadic CRC was defined as that occurring in an individual with no known familial history of a hereditary CRC syndrome and no personal history of a condition associated with an increased CRC risk, namely inflammatory bowel disease.

The chromosomal instability pathway is the most common pathway to sporadic CRC, justifying 84% of the cases analyzed by the Cancer Genome Atlas Network.[19] These tumors are typically microsatellite stable.[19] Microsatellite instability (MSI) results from errors in deoxyribonucleic acid (DNA) replication that go unrepaired in the presence of mutations or epigenetic silencing of the mismatch repair genes. High-grade MSI characterizes CRC arising through another major pathway leading to CRC, the MSI pathway.

Methods

Study population

From February 2011, all consecutive patients with a histological diagnosis of a colorectal adenocarcinoma discussed at the Instituto Português de Oncologia de Lisboa, Francisco Gentil, EPE (IPOLFG) multidisciplinary CRC team meeting who were proposed for surgery were screened for participation. Patients aged 18 years or older who were planned to have a total colonoscopy performed at IPOLFG after study inclusion (either before surgery or during the first year after) were invited to participate in the study, until the planned number of 30 patients was achieved.

Since this was a pilot study, with no available data on the likelihood of finding positive results, a sample of 30 patients was planned based on the central limit theorem, which states that the sampling distribution of any statistic will be normal or nearly normal, if the sample size is large enough. Generally, a sample size of 30 is considered large enough.

Patients were excluded if they were incapable of giving free and informed consent, if there was personal history of inflammatory bowel disease, previous CRC or previous colonic resection surgery or a family history of a CRC hereditary syndrome (familial adenomatous polyposis (FAP), *MUTYH*-associated polyposis, Lynch syndrome (LS), Peutz-Jeghers syndrome or juvenile

polyposis with a genetic-based diagnosis, >100 adenomas, attenuated FAP by de Nielsen et al.[20] criteria, Amsterdam II criteria for LS[21] or World Health Organization (WHO) criteria for hyperplastic polyposis[22]). Patients who were scheduled to receive chemotherapy and/or radiation therapy for the CRC before the surgery and/or the study total colonoscopy were also excluded.

Study design

This was a prospective, observational, single-center study.

At study inclusion, data were collected regarding patients' age and sex, tumor and synchronous lesions' location (in the presence of two or more adenocarcinomas, the largest one at endoscopy was considered the *index* lesion), previous colonic endoscopic exams and/or surgeries and personal or family history (first- and second-degree relatives) of CRC or adenomas and/or other LS tumors,[21] according to the patient and/or accompanying persons and confirmed through medical records whenever possible.

At colonoscopy and/or at the surgical specimen, fresh tissue samples were collected from the tumor, all other macroscopic lesions and flat colonic mucosa at 10–20 cm intervals, from the ileo-cecal valve to the anorectal margin or from the available segments (if the tumor could not be passed at colonoscopy and a segmental resection was performed at surgery). At each segment, five biopsy fragments or equivalent fragments from the surgical specimen were taken. All tubes were labeled with each individual's study number, the type of sample (surgical or from colonoscopy) and the colon position. Samples were kept at −70°C until processing.

All colonoscopies (the first study colonoscopy and all subsequent surveillance exams) were performed by certified gastroenterologists with at least two years' post-training experience. The global cecal intubation rate in our endoscopy unit is 97.5%.

Tumor stage (according to the seventh version of the American Joint Committee on Cancer TNM classification),[23] histological grade, growth at tumor margin, inflammatory infiltrate and histopathology subtypes, according to the WHO classification[24] were recorded.

Molecular and genetic analysis

MSI analysis. Genomic DNA from each tumor and matched normal colonic mucosa was isolated from paraffin-embedded tissue using a proteinase-K digestion method followed by phenol-chloroform extraction.

The MSI status was analyzed using the Bethesda microsatellite markers: BAT26, D17S250, D2S123, BAT25 and D5S346.[25,26] If only one of these markers showed instability, or fewer than five of the markers were amplified, then a second panel of two markers was analyzed (BAT40 and MYCL1).

Each tumor and paired normal DNA was amplified for all markers by polymerase chain reaction (PCR), using fluorescent labeled primers (Applied Biosystems, Foster City, CA, USA) specific for each locus. PCR products were analyzed on an ABI Prism 3130 genetic analyzer using the GeneMapper software (Applied Biosystems). MSI was identified by the presence of additional peaks corresponding to small deletions or insertions in the microsatellite sequences in the tumor DNA when comparing with the paired normal DNA.

Tumors presenting MSI in two or more microsatellite markers were classified as MSI-High (MSI-H), whereas MSI-Low (MSI-L) was defined by the presence of MSI in only one of the respective markers. Tumors without MSI in any of the markers were considered to be microsatellite stable (MSS).

BRAF exon 15 mutation analysis. In brief, genomic DNA isolated from paraffin-embedded tumor tissue was amplified by PCR using the following primers:

5′ TCATAATGCTTGCTCTGATAGGA 3′

5′ GGCCAAAAATTTAATCAGTGGA 3′.

The PCR product was then used for direct sequencing using the Big Dye terminator v1.1 sequencing kit (Applied Biosystems) on an automatic ABI Prism 3130 Genetic Analyzer (Applied Biosystems), in accordance with the manufacturer's instructions.

APC mutation analysis (exon 15). Genomic DNA was extracted from frozen fresh tumor samples and matched macroscopically normal colonic mucosa. DNA was isolated using the genomic DNA purification kit (Citomed), a salting-out-based method, according to the manufacturer's instructions.

Exon 15 (codons 654–1700) mutations were analyzed using the protein truncation test (PTT) as previously reported.[27]

Sequencing analysis. All PTT fragments showing an aberrant electrophoretic banding pattern were sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems) on an automatic ABI Prism 3130 Genetic Analyzer (Applied Biosystems), in accordance with the manufacturer's instructions. Mutation description is according to Genbank NM_000038.5 transcript.

Next-generation sequencing (NGS). *APC* sequence analysis of tumor and macroscopically normal colonic mucosa from three patients was performed using the TruSight Cancer kit (Illumina, San Diego, CA, USA) and the MiSeq Next Generation Sequencer platform

(Illumina) according to the manufacturer's instructions. The data generated were analyzed with the Miseq Reporter v.2.5.1 and ISAAC Enrichment v1.0 and 2.1 software (Illumina) and were visualized using the VariantStudio v2.2 software (Illumina). Data were analyzed only for the *APC* gene.

Statistical analysis

SPSS Statistics 19 (IBM) was used for analysis.

All quantitative variables were summarized through descriptive statistics, namely mean, median, standard deviation and range and qualitative variables though absolute and relative frequencies (as applicable). The association between two categorical variables was tested through the Chi-Square test or Fisher's exact test (as applicable).

Ethics statements

This study received final approval from the ethics committee and IPOLFG Review Board on 3 February 2011. All investigation respected the Helsinki Declaration principles.

Patients were included in the study only after informed consent was obtained.

Results

Thirty patients gave informed consent for the study, but one of them later retracted. In four patients, tissue samples could not be obtained because of the surgery schedule. In one patient, the surgical specimen pathological exam showed a high-grade dysplasia adenoma, with no invasive neoplasia. After these patients were excluded, the final sample consisted of 24 patients (12 female, 12 male), with a mean age of 69.4 years (minimum 50, maximum 87 years). No patients had history of colonic endoscopic exams prior to the ones that led to the CRC diagnosis or of any prior diagnosis of an LS spectrum tumor.

Family history

Six patients had family history of CRC or adenomas – two of them had a single first-degree relative with CRC or adenomas while the remaining four had two or more affected first- or second-degree relatives.

In four patients, the family history was considered high risk (at least three relatives with CRC/adenomas or at least two if one was diagnosed before 60 years of age) and a referral for a familial CRC risk clinic appointment was performed. Three of these patients had MSS tumors and one had an MSI-H tumor. No family fulfilled Amsterdam criteria for LS.

Table 1. Colorectal cancers and synchronous adenomas' locations

	Synchronous adenomas			
	Absent	Right colon	Left colon	Right + left colon
Cancer location				
Right colon	2	4	0	4
Left colon	4	3	3	4

Tumor and synchronous lesions' location

In 10 patients, the tumor was found in the right colon (defined as proximal to the splenic flexure) and 14 patients had sigmoid cancers.

No synchronous CRCs were found.

Synchronous adenomas were found in 18 patients (75%). In 39% of these patients the adenomas were found in the right colon, in 17% the adenomas were found in the left colon or in the rectum, and 44% of patients had adenomas in both halves of the colon.

As shown in Table 1, all patients with right colon cancers who had synchronous adenomas had at least one adenoma in the same colonic half and seven of the 10 patients with left colon cancers who had synchronous adenomas also had at least one in the same colonic half.

Tumor pathological characteristics

Of the 24 patients, one had a mucinous adenocarcinoma and two others had adenocarcinomas with 25–50% mucinous components. These three tumors were all in the right colon ($p = 0.06$) and the mucinous adenocarcinoma was an MSI-H tumor. No other colorectal adenocarcinoma subtypes were found in this series.

In three patients, the CRC had a Crohn-like inflammatory infiltrate – all three three tumors were in the right colon ($p = 0.06$) and they were all MSI-H tumors ($p = 0.03$).

In 17 patients, the tumor had an infiltrative growth margin, while in seven patients there was an expansive growth margin.

The tumors were well differentiated in six patients, moderately differentiated in 15 patients and poorly differentiated in three patients.

There were 13 patients with stage I or II CRCs and 11 patients with stage III or IV CRCs.

Immunohistochemistry and MSI analysis

In one of the cases there were artifactual changes in the tumor tissue and immunohistochemistry analysis could

Table 2. Association between the immunohistochemistry analysis results and the tumor microsatellite instability status

	Microsatellite instability			
	MSS	MSI-L	MSI-H	Fisher's exact test
Immunohistochemistry for DNA repair protein expression				
Normal	12	4	3	$p = 0.007$
Altered	0	0	4	

MSS: microsatellite stable; MSI-L: low-grade microsatellite instability; MSI-H: high-grade microsatellite instability.

Table 3. Association between the tumor location and microsatellite instability status

	Microsatellite instability			
	MSS	MSI-L	MSI-H	Fisher's exact test
Cancer location				
Right colon	4	0	6	$p = 0.047$
Left colon	8	4	2	

MSS: microsatellite stable; MSI-L: low-grade microsatellite instability; MSI-H: high-grade microsatellite instability.

not be performed. Of the remaining cases, three had tumor loss of expression of MLH1 and PMS2 proteins, one had tumor focal loss of expression of MLH1, MSH2 and MSH6 proteins, and in 19 there was MLH1, PMS2, MSH2 and MSH6 protein expression both in the tumor and in the normal tissue.

There was a strong association between an altered immunohistochemistry for DNA repair protein expression and the presence of an MSI-H tumor (Table 2). The tumor in which immunohistochemistry analysis was not performed was also MSI-H.

All four MSI-L tumors were left colon tumors, while MSI-H tumors were significantly more common in the right colon (Table 3).

There was no association between MSI status and patient's sex or age at diagnosis (younger or older than 65), nor with the tumor's grade or stage at diagnosis (data not shown).

The patients with tumor focal loss of expression of MLH1, MSH2 and MSH6 proteins and with the MSI-H tumor in which immunohistochemistry analysis could not be performed were referred for a familial CRC risk clinic appointment, where genetic studies for LS will be proposed.

BRAF *mutation analysis*

In the six patients who had MSI-H tumors with either loss of MLH1/PMS2 expression or unaltered DNA repair protein expression, *BRAF* mutation analysis was performed. A pathogenic somatic mutation was found in two of the patients. Both had right colon cancers, with loss of MLH1/PMS2 expression.

The remaining four patients were referred for a familial CRC risk clinic appointment, where genetic studies for LS will be proposed.

APC *mutation analysis*

APC exon 15 mutation analysis by PTT was performed in all MSS and MSI-L tumors (16 patients) and in three MSI-H tumors with unaltered immunohistochemical analysis for DNA repair proteins and no *BRAF* mutation.

PTT found one exon 15 mutation in seven patients and two *APC* exon 15 mutations in four patients. *APC* mutation analysis was performed by PTT in all available macroscopically normal mucosa samples from patients in whom at least one *APC* mutation was detected in the tumor. No PTT alterations were found in the macroscopically normal mucosa samples in these patients.

All four patients with two *APC* mutations had MSS tumors. Of the seven patients with a single mutation, three had MSS tumors, two had MSI-L and two had MSI-H tumors.

In three patients with equivocal PTT results (possible low-intensity band in the electrophoresis' gel), NGS testing was performed. In one of these patients, in whom PTT had identified one *APC* exon 15 mutation, two additional mutations were found by by NGS – an additional additional exon 15 mutation and an exon 9 mutation. This patient had an MSS tumor.

Some patients in whom *APC* mutations were found in the CRC had synchronous adenomas, but these lesions were never large enough to allow sampling for mutation analysis without compromising the pathological exam. Therefore, *APC* mutation analysis was not performed in synchronous lesions.

Follow-up

After a median follow-up of 30.9 months (3–48 months), four patients had died (three from post-surgical complications, one from heart failure after 37 months' follow-up with no cancer recurrence), two were alive with active disease (one node recurrence, one hepatic recurrence) and 18 were alive and disease free.

After excluding the three patients who died in the post-operative period, six patients (29%) had metachronous adenomas. All six patients with metachronous adenomas had had synchronous adenomas (and four of them had high-risk synchronous adenomas). There was a significant association between the location

Table 4. Location of colorectal cancer (CRC) and adenomas in patients followed after the post-operative period

	CRC and adenomas characteristics				
Patient's sex and age at CRC diagnosis	CRC location	Mucinous component/ Crohn-like infiltrate	MSI status	Synchronous adenomas location	Metachronous adenomas location
Male, 75	Right colon	<25%/Absent	MSI-H	Right colon	NA
Female, 59	Left colon	<25%/Absent	MSI-L	Right+left colon	Right colon
Male, 83	Right colon	<25%/Present	MSI-H	Right colon	Left colon
Male, 58	Left colon	<25%/Absent	MSS	Right colon	Right + left colon
Female, 74	Left colon	<25%/Absent	MSI-L	Left colon	Left colon
Female, 79	Right colon	>50%/Absent	MSI-H	Right colon	NA
Male, 69	Left colon	<25%/Absent	MSS	NA	NA
Female, 79	Right colon	25–50%/Absent	MSS	NA	NA
Male, 57	Left colon	<25%/Absent	MSI-L	Right colon	NA
Female, 79	Left colon	<25%/Absent	MSI-H	NA	NA
Male, 61	Left colon	<25%/Absent	MSI-L	Left colon	NA
Female, 50	Right colon	25–50%/Absent	MSS	Right colon	NA
Male, 51	Right colon	<25%/Present	MSI-H	Right + left colon	NA
Male, 66	Left colon	<25%/Absent	MSS	Right + left colon	Right colon
Female, 75	Left colon	<25%/Absent	MSS	NA	NA
Female, 69	Left colon	<25%/Absent	MSS	Right colon	NA
Male, 70	Right colon	<25%/Absent	MSS	Right + left colon	Right colon
Male, 58	Left colon	<25%/Absent	MSI-H	Left colon	NA
Female, 64	Right colon	<25%/Absent	MSS	NA	NA
Male, 75	Left colon	<25%/Absent	MSS	Right + left colon	NA
Male, 76	Left colon	< 25%/Absent	MSS	Right + left colon	NA

CRC: colorectal cancer; MSS: microsatellite stable; MSI-L: low-grade microsatellite instability; MSI-H: high-grade microsatellite instability; NA: non-applicable.

of synchronous and metachronous adenomas ($p = 0.05$) – all patients who had any right colon metachronous adenomas had previous right colon synchronous adenomas (Table 4). Of the two patients with metachronous adenomas found only in the left colon, one had a previous left colon synchronous adenoma (and a left CRC) and the other patient, who had a previous right colon synchronous adenoma (and a right colon MSI-H cancer), had to undergo a totalization of the colectomy because of two metachronous adenomas, one of which was endoscopically unresectable. This patient is being studied for LS.

Discussion

This small series clearly confirms the fact that there are distinct groups of CRC patients, with different pathways for tumor progression.

As expected, MSI-H tumors were significantly more common in the right colon and significantly more likely to have Crohn-like infiltrates. Adenocarcinomas with more than 25% mucinous components were found only the right colon and the only mucinous adenocarcinoma in the series was a right colon MSI-H tumor. The immunohistochemistry for DNA mismatch repair proteins was also significantly associated with MSI status – all tumors showing any DNA repair protein loss of expression were MSI-H tumors. Taken together, these results reinforce the need to value the pathological exam to stratify CRC patients, namely to select those whose cancer evolved through the MSI pathway. In this series, only two out of six MSI-H patients who were studied had a *BRAF* mutated tumor and the remaining patients are being evaluated for LS. None of these four patients fulfilled revised Bethesda criteria[26] for MSI or DNA repair protein expression analysis, which means four possible LS patients and their families might have been lost – tumors were only tested for MSI because the patients were included in the current study. This is in agreement with the latest common position statement by the American Society of Clinical Oncology and the European Society for Medical Oncology, recommending universal testing of all CRC patients for LS, by MSI or immunohistochemistry analysis, to surpass the lack of sensitivity of all selection criteria.[28]

Regarding tumors that evolve through the chromosomal instability pathway, they mostly correspond to the non-hypermutated CRCs described in the Cancer Genome Atlas Network molecular characterization of sporadic CRC – there was an *APC* gene mutation in around 80% of them and the tumors were CpG islands methylator phenotype (CIMP) negative and mainly MSS.[19]

In the current series, an *APC* gene mutation was found in nine (56%) of MSS or MSI-L tumors, and five of these tumors (all MSS) showed biallelic truncating mutations of the gene. In FAP patients, it was shown that the type of somatic *APC* mutation is determined by the site of the germline mutation.[29] In the presence of certain "first hit" mutations, the "second hit" predominantly occurs through allelic loss in which the allele lost is the "wild-type" one.[29] This mechanism most likely also happens in sporadic CRC and it may partly explain our findings of a high percentage of tumors with a single *APC* mutation. However, another possible explanation is a technical limitation.

Although in the past PTT was considered as the most sensitive method for exon 15 *APC* mutation detection,[29,30] recently it has been shown that NGS and other new ultrasensitive methods are able to detect *APC* mutations previously unnoted by either PTT or Sanger sequencing.[31,32] In the three patients tested by NGS in the present series, a previously PTT undetected exon 15 mutation was found in one patient, which is in accordance with these data.

The fact that we only used PTT for most patients may also account for the fact that, even in those patients with biallelic *APC* mutations, no mutations were found in the macroscopically normal mucosa. Adding to this, there is also a sampling limitation. In each patient, synchronous lesions were never large enough to be sampled and only one sample was collected from each 10 cm colon segment available. In the inflammatory bowel disease setting, authors calculated that to find an alteration present only in a 2 cm diameter patch of colonic mucosa, around 320 random biopsy specimens would be needed.[33]

The association between CRC and synchronous adenomas' location (although not statistically significant) and the statistically significant association between synchronous and metachronous adenomas' location argues in favor of a colonic field defect. The data from this small prospective series are in accordance with our previously published work that showed a significant association between CRC and synchronous adenomas' location in a larger retrospective series.[34]

The small sample size of the current series prevents definitive results and technical limitations also conditioned our ability to directly assess the possibility of a field defect derived from patches of mucosa with a common first mutation. It will remain a hypothesis to be tested in the future. However, the main advantage of this series is that it corresponds to a non-selected sporadic CRC patient sample in which the notion of different pathways to cancer and the possibility of a colonic patchy field defect could be prospectively tested and confirmed.

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Conflicts of interest

Isadora Rosa acted as a consultant or speaker at scientific meetings sponsored by MSD, AbbVie, Dr Falk Pharma and Ferring. She received support to participate in scientific meetings by MSD, AbbVie, Dr Falk Pharma, Ferring, Hospira and Norgine.

The other authors have nothing to declare.

Author contributions are as follows:

Isadora Rosa: Coordinating/Lead investigator: Planned and designed the study, selected patients, performed all study colonoscopies, collected and analyzed data and wrote the final report.

Paulo Fidalgo: Planned and designed the study, collaborated in data analysis and in the writing of the final report. Approved the final draft submitted.

Bruno Filipe: Performed the molecular analysis and collaborated in data analysis and in the writing of the final report. Approved the final draft submitted.

Cristina Albuquerque: Participated in the design of the study and collaborated in data analysis and in the writing of the final report. Approved the final draft submitted.

Ricardo Fonseca: Collected surgical specimen samples, performed the pathological exam of samples and the immunohistochemistry analysis and collaborated in data analysis and in the writing of the final report. Approved the final draft submitted.

Paula Chaves: Collected surgical specimen samples, performed the pathological exam of samples, participated in the design of the study and collaborated in data analysis and in the writing of the final report. Approved the final draft submitted.

A. Dias Pereira: Participated in the design of the study and collaborated in data analysis and in the writing of the final report. Approved the final draft submitted.

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5. Discussão e Conclusões

Frank e Nowak propuseram, em 2003, que a maioria dos cancros manifestados na vida adulta em tecidos epiteliais, como o do cólon, tem origem numa linhagem derivada de uma célula estaminal mutada numa fase precoce do desenvolvimento.[73] Neste tipo de tecidos, a população celular expande-se exponencialmente no início da vida para formar o órgão, cuja constante renovação na vida adulta é assegurada pela divisão controlada, homeostática, das células estaminais. A taxa de ocorrência de mutações numa célula estaminal adulta, que se divide com menor frequência, parece ser muito menor que a taxa de mutações durante a fase exponencial do desenvolvimento. Assim, a probabilidade de um cancro decorrer de uma primeira mutação somática adquirida precocemente será muito significativa.[73,74] Vários modelos matemáticos, aplicados quer a dados populacionais da incidência global de CCR,[75] quer a dados de incidência estratificados pelo *status* de MSI do CCR,[76] apontam para uma sequência necessária de 4 a 8 mutações até à manifestação clínica do cancro e para uma elevada probabilidade de que uma ou duas destas mutações ocorram durante o desenvolvimento embrionário.[40,75,76] Todos estes modelos implicam um mosaicismo cólico - o cólon adulto é um conjunto de retalhos de mucosa derivados de diferentes células estaminais embrionárias e um número variável destas células pode ter adquirido uma ou mais mutações durante a gestação, mutações essas presentes em todo o respetivo retalho.

No artigo Adenoma incidence decreases under the effect of polypectomy, demonstramos que numa população de risco padrão de CCR a incidência de adenomas não aumenta exponencialmente com a idade, como defendido no passado.[77,78] De facto, depois de atingido um pico de incidência, se os adenomas forem removidos na totalidade por polipectomia, a taxa de aparecimento de novos adenomas parece tender, ao longo do tempo, para o zero. Esta distribuição está de acordo com um modelo de carcinogénese em que a primeira mutação ocorre sempre na embriogénese e a acumulação necessária de eventos subsequentes vai resultar numa manifestação fenotípica, o adenoma, que ocorre apenas numa determinada janela temporal.

Na literatura, também vários estudos têm vindo a demonstrar que uma colonoscopia basal de rastreio (ou seja, realizada aos 50 anos num indivíduo com risco padrão para CCR) sem adenomas, ou apenas com 1-2 adenomas sem caraterísticas de risco só precisa de ser repetida após 10 anos.[79,80] O risco quer de CCR quer de adenomas de risco neste período é baixo (<5%)[79] e é seguro manter intervalos de vigilância com colonoscopia total de 10/10 anos se nos exames nunca existirem critérios de risco.[79,80] Aos 75-85 anos, a vigilância pode ser suspensa em indivíduos sem adenomas avançados, porque os riscos da colonoscopia excederão os seus benefícios.[79,80]

Mesmo em indivíduos com adenomas de risco, tem também vindo a ser demonstrado que após a sua adequada remoção, se nos exames de vigilância não surgirem mais destas lesões, os intervalos de vigilância podem ser alargados.[79,80]

Todas estas constatações, que conduziram aos atuais programas de vigilância recomendados em todo o mundo desenvolvido, estão de acordo com a noção que defendemos, de uma janela temporal de risco mais elevado para adenomas/CCR, após a qual o risco vai diminuindo ao longo do tempo. Esse risco vai extinguir-se quando se esgotar a população de células cometida precocemente à formação de adenomas – população essa que é finita e suscetível de ser erradicada, através das polipectomias.

Se o principal condicionador do CCR esporádico não fosse uma predisposição genética precoce, mas sim a acumulação de agressões ambientais, associada ou não ao envelhecimento celular e à consequente perda de fidelidade na replicação do DNA, o risco de adenomas e CCR tenderia necessariamente a aumentar sempre com o tempo, com a necessidade de manter indefinidamente, ou até encurtar, os intervalos de vigilância para assegurar a eficácia dos programas.

Os estudos em populações de emigrantes do Japão para os Estados Unidos da América (EUA) realizados entre 1960-1970, que mostraram um aumento significativo da incidência de CCR na 1ª e 2ª gerações de emigrantes, quando comparadas com a população de origem, constituíram a base para a teoria ambiental do CCR esporádico.[81] No entanto, dados mais recentes mostram claramente que, no que diz respeito a populações de migrantes asiáticos para os EUA, enquanto a incidência de CCR aumenta claramente nos descendentes de migrantes japoneses, esta incidência diminui ligeiramente nos migrantes chineses e não se altera nos migrantes filipinos.[82] Os havaianos de origem japonesa têm atualmente a maior incidência de CCR do mundo, ou seja, não só superior à dos japoneses que permanecem no país de origem, mas também muito superior à dos americanos de raça caucasiana.[82] Em conjunto, estes dados não apontam para um papel fundamental dos fatores ambientais na origem do CCR, mas sim para uma predisposição genética claramente distinta entre populações de origens geográficas diversas, que se manifesta consoante os fatores ambientais presentes[82] – estes constituem-se como fatores promotores e não iniciadores do CCR esporádico.

Recentemente, foi possível conciliar os modelos de evolução clonal e da célula estaminal[83] na explicação da origem do CCR com a constatação dos fenómenos de sucessão no nicho, em que determinadas mutações, nomeadamente no gene *APC*, conferem vantagem seletiva à célula estaminal.[35] Da mesma forma, o papel promotor dos fatores ambientais pode conciliar-se, neste modelo, com a evidência de que o destino (permanecer como célula estaminal *vs* migrar para a «*transit-amplifying zone*») de cada uma das células resultantes da divisão de uma célula estaminal pode ser modulado por sinais enviados pelas células que rodeiam a cripta e que estes sinais podem variar, por exemplo, com a ingestão calórica.

Ficando claro que a larga maioria dos dados aponta para uma predisposição genética como fator iniciador do CCR, a noção de que esta predisposição não ocorre de forma uniforme ao longo do cólon também acaba por ficar reforçada pelos estudos de migrantes. Mesmo na população japonesa, em que se verificou um aumento do risco de CCR com a migração para os EUA, esse aumento de risco não foi uniforme para todas as localizações de CCR – o excesso de risco quando os descendentes de japoneses foram comparados com a população americana

caucasiana só se verificou para o recto e cólon esquerdo.[82] Este é mais um dado a favor da ideia de um mosaicismo cólico.

No artigo The co-localization of carcinomas and adenomas favors a regional field defect in the colon: an observational study, foi possível demonstrar, em doentes com CCR e adenomas síncronos, uma correlação estatisticamente significativa entre a localização do CCR e dos adenomas, sobretudo para adenomas de risco. A probabilidade de deteção de adenomas em cada metade do cólon era significativamente superior quando o CCR se localizava nessa mesma metade. Com o artigo Sporadic Colorectal Cancer: studying ways to an end foi possível completar estes resultados, demonstrando também uma co localização, com significado estatístico, dos adenomas síncronos e metacrónicos. Estes dados favorecem a hipótese de um primeiro evento comum, presente apenas nalguns retalhos cólicos, onde as lesões vão depois surgir numa dada janela temporal.

Até ao presente, mesmo nos estudos que evidenciam a presença de mosaicismo cólico em diferentes contextos, nunca foi possível demonstrar claramente qual o primeiro evento.[59-61]

No que diz respeito aos CCRs que evoluem pela via da instabilidade cromossómica, há evidência acumulada de que a mutação do primeiro alelo do gene *APC* é maioritariamente o primeiro evento na sequência de carcinogénese. O facto de não ter sido possível demonstrar um mosaicismo com base neste primeiro evento no nosso trabalho Sporadic Colorectal Cancer: studying ways to an end pode simplesmente dever-se a dificuldades práticas. Estas dificuldades vão desde o tamanho da amostra às limitações da técnica de análise de mutações, passando pela quase inevitável impossibilidade de amostragem de todo o cólon. No que diz respeito à deteção de mutações no gene *APC*, mesmo o *Next-Generation Sequencing*, habitualmente utilizado na pesquisa de mutações germinais, tem um limiar de deteção que pode ser insuficiente para identificar mutações somáticas presentes apenas num número possivelmente muito reduzido de células.

A avaliação do papel destas limitações nos resultados passa por, num trabalho futuro, testar esta hipótese numa amostra maior, procurar testar lesões adenomatosas síncronas e, eventualmente, usar técnicas ainda mais sensíveis.

Outro campo em que a noção de defeito de campo cólica pode ser mais explorada é o da DII, onde será de esperar encontrar padrões de expressão génica e/ou epigenética distintos entre lesões displásicas secundárias à inflamação crónica e lesões adenomatosas esporádicas, que derivam de células em que ocorreu uma primeira mutação na embriogénese.

Em suma, demonstrámos, sob efeito da polipectomia, uma diminuição da incidência de adenomas ao longo do tempo, que favorece a noção da eliminação de clones derivados de células mutadas precocemente. Demonstrámos também uma co localização de CCR e adenomas síncronos e de adenomas síncronos e metacrónicos, que aponta para um padrão de defeito de campo segmentar no cólon – o mosaicismo cólico. Não foi possível identificar a base genética deste mosaicismo, possivelmente por limitações técnicas, mas a evidência na literatura aponta neste sentido e parece-nos que prosseguir no estudo da origem do CCR será o caminho para a sua eliminação.

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«The crypt bottom harbors slender, cycling
"crypt base columnar" (CBC) cells [31], which
were historically proposed to represent intestinal
stem cells [32] (Fig. 3A). Exploiting the
expression of Wnt target gene Lgr5 in CBCs,
genetic labeling of Lgr5+ crypt cells indeed demonstrated
that these long-lived cells generate all
differentiated intestinal cell types [25]. Therefore,
CBCs constitute multipotent intestinal stem cells[25]
that require Wnt for proliferation [27, 33],
perhaps explaining why Wnt is crucial for intestinal renewal.»

Hans Clevers *et al*.